THE MOLECULAR BIOLOGY OF ACUTE PROMYELOCYTIC LEUKAEMIA AND ITS RESPONSE TO THERAPY

by

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ABSTRACT

Acute promyelocytic leukaemia (APL) is characterised by the t(15;17) leading the formation of PML-RARα and RARα-PML fusion genes. This rearrangement predicts a favourable differentiation response to all-transretinoic acid (ATRA), which confers improved survival when combined with chemotherapy. Therefore, establishing the presence of the t(15;17) at the molecular or cytogenetic level is critical for optimal management of patients with this disease.

This thesis evaluated RT-PCR and PML-immunofluorescence techniques as a means of identifying underlying PML/RARα rearrangements in cases of suspected APL entered into the MRC ATRA trial. These approaches revealed that the classic t(15;17) is not identified cytogenetically in 15% cases with PML/RARα rearrangements. Further characterisation of such cases showed that PML-RARα was the sole fusion gene formed as a result of cytogenetically cryptic rearrangements, thereby supporting its product as the key oncogenic fusion protein. Immunofluorescence studies demonstrated a close correlation between expression of PML-RARα and delocalisation of PML from nuclear body structures; whereas a normal PML staining pattern was found in APL with a PLZF/RARα rearrangement. This showed that delocalisation of PML from nuclear bodies is not a prerequisite for the pathogenesis of APL, or indeed a final common pathway to leukaemogenesis in cases with translocations involving fusion partners other than PML. In addition, nested RT-PCR was also used to define targets for minimal residual disease monitoring, which was shown to provide an independent prognostic variable.

Another aspect of this study was the investigation of mechanisms underlying expression of the T cell marker CD2 in APL. DNase I hypersensitivity assays established an identical pattern of hypersensitive sites in the region flanking the CD2 locus in APL samples and myeloid cell lines irrespective of CD2 status, to that detected in T cells. This suggests that regulatory regions of CD2 may be accessible to DNase I during normal myelopoiesis, and that the chromatin configuration and the CD2 expression levels associated with APL blasts could reflect the nature of the haemopoietic progenitors targeted by the PML/RARα rearrangement.
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ABBREVIATIONS

ALL                acute lymphoblastic leukaemia
ABMT               autologous bone marrow transplant
alloBMT            allogeneic bone marrow transplant
AML                acute myeloblastic leukaemia
AMV                avian myeloblastosis virus
APL                acute promyelocytic leukaemia
ATRA               all-trans-retinoic acid
BMT                bone marrow transplant
BSA                bovine serum albumin
CCR                continuous complete remission
CML                chronic myeloid leukaemia
CNS                central nervous system
CR                 complete remission
CSF                cerebrospinal fluid
DEPC               diethyl pyrocarbonate
DMSO               dimethyl sulphoxide
dNTPs              deoxynucleotide triphosphates
EDTA               ethylene diamine tetra acetic acid
FAB                French American British leukaemia classification
FCS                fetal calf serum
FISH               fluorescence in situ hybridisation
FITC               fluorescein isothiocyanate
HCl                hydrochloric acid
H2O                water
KCl                potassium chloride
KH2PO4             potassium di-hydrogen orthophosphate
MgCl2              magnesium chloride
MRD                minimal residual disease
NaCl               sodium chloride
NADPH              reduced nicotinamide adenine dinucleotide
Na2HPO4            phosphate
NaH2PO4            di-sodium hydrogen orthophosphate
NaOAc              sodium di-hydrogen orthophosphate

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NBT  nitroblue tetrazolium
NaOH  sodium hydroxide
OD   optical density
OLB  oligo labelling buffer
PBSCT peripheral blood stem cell transplant
PCR  polymerase chain reaction
PE   phycoerythrin
PLL  prolymphocytic leukaemia
PNK  polynucleotide kinase
RA   retinoic acid
RAR  retinoic acid receptor
RARE retinoic acid response element
rpm  revolutions per minute
RT   reverse transcription
RT-PCR reverse transcriptase polymerase chain reaction
RXR  retinoid X receptor
SDS  sodium dodecyl sulphate
TR   thyroid hormone receptor
TPA  12-O-tetradecanoylphorbol 13-acetate
UV   ultraviolet
VDR  vitamin D receptor
v/v  volume for volume
WCP  whole chromosome paint
w/v  weight for volume
Publications arising from work included in this thesis


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To Kate Grimwade,

We are all sorry you are not around to see this thesis completed.
Acute promyelocytic leukaemia (APL) presents a unique example of a disease for which a successful drug therapy, in the form of all-trans retinoic acid (ATRA), has been developed that directly addresses and overcomes the underlying molecular abnormality. For well over a decade, retinoids have been noted to possess therapeutic activity which is virtually specific to the acute promyelocytic form of acute myeloid leukaemia (AML) (Breitman et al., 1981). Subsequent clinical trials have shown that ATRA can achieve remission rates comparable to conventional chemotherapy in newly diagnosed APL, but in addition demonstrated benefit in a series of patients previously resistant to chemotherapy or treated in relapse (Huang et al., 1988; Castaigne et al., 1990; Chomienne et al., 1990). Parallel in vitro studies revealed that remission is mediated through terminal differentiation of the leukaemic clone rather than by a cytotoxic effect (Huang et al., 1988; Castaigne et al., 1990; Chomienne et al., 1990); this was subsequently confirmed by clonal analysis of APL blasts and peripheral blood neutrophils following ATRA therapy (Elliott et al., 1992).

Since the late 1970's it has been appreciated that APL is characterised by the reciprocal translocation, t(15;17)(q22;q21), present in the majority of cases (Rowley et al., 1977; Larson et al., 1984). However, it was not until 1990 that the APL breakpoint region was ultimately cloned, and the significance of the response to retinoids noted in early clinical studies became apparent (Borrow et al., 1990; de Thé et al., 1990). The translocation was found to disrupt a previously uncharacterised gene, PML on chromosome 15 and the retinoic acid receptor-α gene (RARα) on 17. RARα belongs to the steroid hormone receptor superfamily and acts as a transcription factor mediating the effect of retinoic acid (RA) at specific response elements (Giguere et al., 1987; Leid et al., 1992a). The translocation leads to the formation of PML-RARα and RARα-PML chimaeric genes. PML-RARα derived from add(15q) retains key functional domains of both respective fusion partners and hence has been considered to play a key role in leukaemogenesis, whilst the significance of reciprocal RARα-PML transcripts which are detected in approximately 75% of cases (Alcalay et al., 1992; Borrow et al., 1992; Grimwade et al., 1996; Li et al., 1997a) remains unclear.
The realisation that the PML-RARα fusion protein retains the RA ligand binding domain, suggested that it may not only mediate leukaemogenesis, but in some way account for the unique sensitivity of APL to differentiation by retinoids such as 9-cis RA and ATRA. Therefore over the last few years research has been aimed at determining the normal function of PML, the role of retinoid receptors in haemopoietic differentiation, how the rearrangement of PML and RARα might promote leukaemogenesis, the mechanisms by which ATRA reverses the leukaemic phenotype and finally at establishing the role of molecular analyses as a means of directing treatment approach in patients with APL.

1.1. Clinical features of acute promyelocytic leukaemia

1.1.1 Morphological features

APL is one of the commoner forms of AML, accounting for approximately 10% of de novo cases, and typically occurs in early middle age (Stone & Mayer, 1990; Avvisati et al., 1992). The disease is believed to represent a clonal expansion of haemopoietic precursor cells associated with a differentiation block in myeloid development that leads to an accumulation of abnormal promyelocytes in the marrow (Grignani et al., 1993; 1994). This appearance has led to the designation of APL as AML M3 within the French-American-British (FAB) morphological classification, which takes into account the presumed lineage and degree of differentiation of the leukaemic population (Bennett et al., 1976). Despite the presence of an underlying PML/RARα rearrangement in the majority, there is some degree of morphological heterogeneity amongst cases classified as M3, including a hypergranular ("classical") group (Bennett et al., 1976), a hypogranular or microgranular ("variant" or M3v) category (Golomb et al., 1980; Bennett et al., 1980) which also encompasses a rarer hyperbasophilic form (McKenna et al., 1982); whilst more recently a hypereosinophilic subtype of the disease has been described (Yu et al., 1997). The majority of APL cases present with "classical" morphology, whereby the marrow is replaced by hypergranular promyelocytes including characteristic "faggot cells" containing numerous Auer rods. Patients with this form of the disease are typically pancytopenic at the time of diagnosis with scanty abnormal cells detected in the peripheral blood film (Bennett et al., 1976). Whereas in M3v, which accounts for approximately 15% of APL cases (Stone & Mayer, 1990), patients typically present with leucocytosis characterised by the presence of leukaemic cells with bilobed
Chapter one: Introduction

("cottage loaf" shaped) nuclei and which appear less heavily granulated than those of classical APL by light microscopy with conventional May-Grünwald-Giemsa staining (Golomb et al., 1980; Avvisati et al., 1992). Nevertheless, electron microscopy and immunocytochemistry reveal that the leukaemic cells still contain numerous granules which are smaller than those of classical APL, (Golomb et al., 1980), and account for the characteristically intense myeloperoxidase and sudan black staining of leukaemic blasts associated with all forms of the disease (Bain, 1990). The peripheral blood picture of M3v may be confused with that of myelomonocytic or monocytic/monoblastic leukaemias (AML M4/5), although in most instances these distinct disease entities may be distinguished by immunocytochemistry, immunophenotyping and cytogenetics (Bain, 1990), without necessarily resorting to molecular diagnostic techniques. Interestingly, the leukaemic population within the bone marrow in M3v cases is typically more granular than that observed in the blood, although faggot cells are rare or absent. (McKenna et al., 1982). This raises the possibility that the division of APL into classical and variant morphological subgroups may be somewhat artificial, as they may actually represent extreme ends of a spectrum. Indeed, the morphological appearances may be interchangeable, since patients with classical APL may demonstrate reduced granularity on relapse, whereas culture of variant cells may be associated with an increase in granularity more typical of classical morphology (Berger et al., 1981; Castoldi et al., 1994).

1.1.2 Aetiology of the coagulopathy associated with APL

The heavy granulation associated with APL cells has been considered to account for the severe haemorrhagic problems that typify the disease (Linch et al., 1994). The potentially life threatening bleeding diathesis is multifactorial, reflecting to varying degrees disseminated intravascular coagulation, precipitated by release of procoagulant substances from blasts and possibly endothelium; hyperfibrinolysis secondary to release of plasminogen activators and proteolysis, compounded by thrombocytopenia. (Avvisati et al., 1992; Tallman & Kwaan, 1992; Linch et al., 1994; Barbui et al., 1998). Furthermore, the coagulopathy may be exacerbated on commencement of chemotherapy, presumably due to release of procoagulant and fibrinolytic factors as APL cells become disrupted (Tallman & Kwaan, 1992), accounting for a significantly greater induction death rate as compared to many other forms of AML (Grimwade et al., 1998a). ATRA, associated with remission induction by differentiation rather than cell lysis, has been found to ameliorate the coagulopathy (Castaigne et al., 1990), leading to a rapid
improvement in hyperfibrinolysis/proteolysis, although the excess procoagulant activity leading to thrombin generation may be more persistent raising concerns that it could increase the incidence of thromboembolic complications (Rodeghiero & Castaman, 1994).

1.1.3 Prognosis of APL and the role of ATRA therapy

Despite the risk of early mortality secondary to the bleeding diathesis, a number of studies using conventional chemotherapy have demonstrated that APL represents a relatively favourable prognostic group of AML (Keating et al., 1988; Samuels et al., 1988; Swansbury et al., 1994; Grimwade et al., 1998a). This reflects low levels of primary drug resistance in addition to a relatively low risk of relapse. Remission induction achieved with ATRA, in contrast to chemotherapeutic agents, does not induce marrow aplasia; due to its differentiating effect on the leukaemic clone with a probable concomitant stimulation of normal colony forming activity (Sakashita et al., 1993). Marrow aplasia increases the risk of death from haemorrhage and infection. Hence, it was hoped that initial treatment of APL with ATRA, by both ameliorating the coagulopathy and achieving remission without marrow aplasia, might lead to further improvements in complete remission (CR) rates and hence overall survival. However, it is now clear that early benefits of ATRA may be offset by morbidity or mortality due to a constellation of clinical features known as the "ATRA syndrome" that develop in up to 30% of patients (Frankel et al., 1992). This phenomenon may reflect the modulation of cell surface markers as differentiation occurs (Di Noto et al., 1994) and leads to fluid retention associated with pleural and pericardial effusions and pulmonary infiltrates which can be fatal (Frankel et al., 1992); although this may be averted if steroids are started promptly (Vahdat et al., 1994). A further problem with the use of ATRA as a single agent therapy is that it does not lead to the eradication of the disease related clone: hence responses are not durable without consolidation chemotherapy (Lo Coco et al., 1992; Miller et al., 1992). In the majority of cases, resistance to ATRA most likely reflects a rapid reduction in circulating drug levels due to induction of cytochrome p450 and cellular retinoic acid binding proteins (CRABPs), which cannot be overcome by dose escalation (Warrell, 1993). This pharmacokinetic phenomenon has stimulated interest in alternative retinoid agents that have activity in APL, such as 9-cis RA (Fenaux et al., 1997a) and Am80 (Tobita et al., 1997) which have been found to induce remissions in some patients relapsing following ATRA. More recently, it has been shown that a subgroup of patients who have previously been exposed to ATRA, relapse
with a leukaemic clone that has acquired a mutation within the ligand-binding domain of PML-RARα which is associated with clinical resistance to further ATRA therapy (Imaizumi et al., 1998; Ding et al., 1998). The frequency of this phenomenon amongst patients induced simultaneously with ATRA and chemotherapy remains to be determined. However, this finding clearly has implications for the most appropriate treatment of patients relapsing following first line therapy. This could include arsenic compounds such as AS₂O₃ and Realgar (Shen et al., 1997; Lu et al., 1997) which have recently been shown to be highly efficacious in the treatment of APL. The optimal place of these agents, which appear to induce clinical remission through induction of apoptosis rather than differentiation of the leukaemic clone (Zhu et al., 1997; Wang et al., 1998c), in the management of patients with APL remains to be determined.

Whilst the early promise of ATRA as a single agent therapy for APL was not ultimately fulfilled, subsequent studies have demonstrated that a combined treatment approach using ATRA with chemotherapy led to a significant improvement in survival in patients with APL compared to chemotherapy alone (Fenaux et al., 1994; Kanamaru et al., 1995; Tallman et al., 1997; Burnett et al., 1997). The optimal timing and duration of ATRA therapy remains to be determined. However, the recent MRC ATRA trial demonstrated superior remission rates, reduced relapse risk and improved overall survival in patients receiving prolonged ATRA therapy simultaneously with induction chemotherapy in comparison to those treated with a short 5 day course of ATRA prior to chemotherapy and also to a historical control group treated with identical chemotherapy, but without ATRA (Burnett et al., 1997). Similarly other large multicentre trials have demonstrated extremely favourable results with protocols combining simultaneous prolonged ATRA and chemotherapy as induction treatment for APL, associated with low rates of ATRA syndrome (Burnett et al., 1997; Mandelli et al., 1997). Interestingly the US Intergroup study suggested that ATRA could also be of benefit as maintenance therapy as means of reducing relapse risk (Tallman et al., 1997). As to whether this is also the case for patients previously exposed to prolonged courses of ATRA as induction therapy, particularly in the light of the pharmacokinetic considerations and potential acquisition of PML-RARα ligand binding domain mutations, also remains to be determined.

This thesis will in part address the role of molecular diagnostic techniques as a means of confirming a clinical diagnosis of APL. It is clear that this condition requires a specific treatment approach, particularly in relation to the coagulopathy which carries a high risk of induction death unless effectively addressed, and also with regards the judicious use of retinoids which confer significant improvements in overall survival rates. Since the
presence of the \textit{PML/RAR\alpha} rearrangement essentially defines the subgroup of patients most likely to gain a beneficial response to retinoids (Miller \textit{et al.}, 1992; Licht \textit{et al.}, 1995; Mozziconacci \textit{et al.}, 1998), it is clear that establishing the presence of this rearrangement is critical not only for optimal patient management, but also for meaningful analysis of clinical trials involving retinoids.

1.2. Molecular characterisation of the breakpoint regions associated with the t(15;17)

1.2.1 Identification of the genes disrupted by the t(15;17)

The APL breakpoint region was initially successfully characterised by three different groups virtually simultaneously, using two distinct methodological approaches. Borrow \textit{et al.} (1990) employed a physical mapping strategy; identifying clones generated from a Not I linking library constructed from an interspecies hybrid containing 17q as its only human material, which were found to flank the APL breakpoint region. Ultimately, screening an HL60 cDNA library revealed that the gene rearranged on 17q was \textit{RAR\alpha}; with disruption occurring within the second intron (numbering system of Brand \textit{et al.}, 1990). \textit{RAR\alpha} was screened independently as a candidate gene by other groups; knowing its proximity to the APL breakpoint region on 17q and bearing in mind the unique sensitivity of the disease to retinoids such as ATRA which had been established previously (Longo \textit{et al.}, 1990; de Thé \textit{et al.}, 1990; Alcalay \textit{et al.}, 1991).

\textit{RAR\alpha} was found to be fused to a novel gene on chromosome 15q; initially known as myl and subsequently renamed \textit{PML} (for ProMyelocytic Leukaemia) (de Thé \textit{et al.}, 1990; Alcalay \textit{et al.}, 1991; Goddard \textit{et al.}, 1991; Kakizuka \textit{et al.}, 1991; de Thé \textit{et al.}, 1991). Characterisation of PML led to the identification of a number of motifs, initially suggesting a role as a putative transcription factor-see Figure 1.1 (Kakizuka \textit{et al.}, 1991;de Thé \textit{et al.}, 1991; Goddard \textit{et al.}, 1991). The N-terminal region of PML is proline rich as previously found in the transcriptional activation domain of CTF (Mermod \textit{et al.}, 1989) and in a number of other transcription factors (Mitchell & Tjian, 1989). Adjacent to this region are 3 cysteine-rich motifs, followed by a coiled-coil domain (Kakizuka \textit{et al.}, 1991; de Thé \textit{et al.}, 1991; Goddard \textit{et al.}, 1991; Freemont, 1993). The most N-terminal of these domains has a C\textsubscript{3}H\textsubscript{C}4 configuration of cysteine and histidine residues, homologous to the zinc finger motif first identified in RING 1
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(Freemont et al., 1991). This motif, which has subsequently been identified in an ever increasing number of proteins, including the predicted product of the \textit{BRCA1} breast cancer susceptibility gene (Miki et al., 1994), has become known as the RING-finger (Freemont, 1993; Saurin et al., 1996). This domain, together with the two adjacent zinc binding cysteine/histidine-rich regions, known as "B boxes" and the neighbouring coiled-coil domain comprise the RBCC or "tripartite" motif (Freemont, 1993; Saurin et al., 1996). This motif is also present in two other RING family members, T18 (Miki et al., 1991) and RET (Takahashi et al., 1988), which interestingly are involved in oncogenic fusion proteins (Kastner et al., 1992; Freemont, 1993). This would suggest that these motifs, which are putative interfaces for protein-protein interactions, may play an important role in the transformation potential of these proteins. Towards the C-terminus of PML, is the putative nuclear localisation signal (Flenghi et al., 1995), and a serine/proline-rich region which has been considered a potential site of phosphorylation by casein kinase II (Kakizuka et al., 1991; Kastner et al., 1992).
### Figure 1.1

Schematic representation of the fusion products generated by the t(15;17) in APL, associated with the commonest PML breakpoint sites (3' = bcr 1 & 5' = bcr 3). Adapted from Goddard et al., (1991); Pandolfo et al., (1992); Alcalay et al., (1992).
Figure 1.2

Schematic representation of the genomic organization at the chromosome 15 and 17 breakpoint regions in a case of APL associated with a 3' (bcr 1) PML breakpoint. The limits of other potential PML breakpoints (bcr 2 and bcr 3) as defined by Southern blotting are denoted by double-ended horizontal arrows. Bcr 1 breakpoints are restricted to intron 6, and PML breakpoints of virtually all bcr 2 and bcr 3 cases fall within exon 6 or intron 3 respectively, as indicated by the vertical arrows. Therefore, bcr 3 breakpoints generally lead to the translocation of PML exons 4 to 9 to chromosome 17. Exons 4 to 9 of PML are subject to alternative splicing, generating multiple PML-RARα and RARα-PML fusion products and up to 13 PML isoforms. Figure adapted from Alcalay et al., (1992), Borrow et al., (1992), Goddard et al., (1991), Pandolfi et al., (1992).
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By comparison of the predicted amino-acid sequence of RARα with the functional regions characterised in steroid and thyroid hormone receptors, it was apparent that it can be divided into 6 functional domains (Giguere et al., 1987; Leid et al., 1992a). At the amino-terminus is the A/B domain which is considered to have a ligand-independent transactivation function (designated AF-1) (Leid et al., 1992a), modulating and augmenting the relatively more important ligand-dependent transactivation function (AF-2) mediated by carboxy-terminal regions of the receptor (Nagpal et al., 1992; 1993). Studies using truncated retinoic acid receptors (RARs) have demonstrated that the functional activity attributable to the amino-terminal region varies considerably depending upon the response element and promoter employed (Nagpal et al., 1992). This suggests that the A/B domain may influence the pattern of retinoid responses, which is of interest, considering that the disruption of RARα in APL occurs between these two domains (Borrow et al., 1990). The adjacent C domain contains 2 zinc finger motifs which are required for DNA binding; the stem of the most N-terminal finger confers specificity of the interaction, lying within the major groove of DNA (Luisi et al., 1991; Schwabe et al., 1993). In steroid hormone and retinoid receptors, the adjacent D domain may provide a nuclear localisation signal (Beato, 1989). The E domain contains the ligand binding region and confers the ligand dependent transactivation function (AF-2) (Leid et al., 1992a; Beato, 1989). AF-2 reflects displacement of corepressors from this region of the receptor following ligand-binding, accompanied by interaction with coactivators, as discussed in more detail below (reviewed Chambon, 1996). Furthermore, the E domain possesses a series of hydrophobic heptad repeats which form an important dimerisation domain, mediating the interaction between retinoic acid receptors (RARs) and retinoid-X receptors (RXRs) (Leid et al., 1992a,b; Danielian et al., 1992; Durand et al., 1994). The role of the C-terminal F domain of RARs is not clear, particularly as it is absent in the RXRs (Leid et al., 1992b).

1.2.2 Fusion products generated by the t(15;17)

The 15;17 translocation leads potentially to the formation of 3 different abnormal products: PML-RARα derived from add(15q), RARα-PML from del(17q) and aberrant truncated PML—see Figure 1.1 (de Thé et al., 1990; Goddard et al., 1991; de Thé et al., 1991; Kakizuka et al., 1991; Pandolfi et al., 1992; Borrow et al., 1992; Alcalay et al., 1992). The PML-RARα product involves fusion of the N-terminal portion of PML, including the proline rich region and "tripartite motif" to the B domain of RARα, hence incorporating DNA binding, ligand binding and heterodimerisation motifs of the
receptor. RARα-PML retains only the RARα transactivation domain (A) and variable C-terminal portions of PML that may include the serine/proline rich potential phosphorylation site (Alcalay et al., 1992; Borrow et al., 1992). Aberrant C-terminally truncated PML proteins which can lack the coiled-coil and second B box domain can also result from the translocation (Pandolfi et al., 1992; Fagioli et al., 1992), and could potentially disrupt wild type PML function and hence play a contributory role in leukaemogenesis. However, since PML-RARα retains key functional domains of both respective fusion partners, it has always been considered the most likely mediator of leukaemic transformation.

1.2.3 Characterisation of breakpoint regions within RARα and PML associated with the t(15;17)

The APL breakpoint on chromosome 17 invariably occurs within the RARα second intron which covers at least 10kb (Borrow et al., 1990; Pandolfi et al., 1992). Little is known as to the mechanisms underlying the t(15;17), however a small study has suggested clustering of breakpoints within the intron, in a region of only 50bp associated with a high frequency of topoisomerase I and II sites. An in vitro transfection-recombination assay suggested that this region may represent a hot spot for illegitimate recombination (Tashiro et al., 1994). In contrast to RARα in which breakpoints occur within a single intron, two major breakpoint regions have been delineated within PML. In approximately a third of patients a 5’ breakpoint occurs (bcr 3), usually within intron 3 (Pandolfi et al., 1992; Biondi et al., 1992). This leads to the formation of a fusion product comprising the N-terminal 372 amino-acids of PML linked to the C-terminal 403 residues of RARα (Goddard et al., 1991; Kakizuka et al., 1991), predicting a 90kD protein. In the remaining two thirds of cases the PML breakpoint occurs more 3’ (Biondi et al., 1992); in the majority of APL patients and in the APL cell line NB4 (Lanotte et al., 1991) the breakpoint lies within intron 6 (bcr 1), whereas in approximately 6-7% of patients disruption occurs more proximally (bcr 2), typically within exon 6 (Pandolfi et al., 1992; Gallagher et al., 1995), see Figure 1.2. The bcr 1 pattern leads to the fusion of the N-terminal 530 amino-acids of PML to RARα predicting a protein of 105kD (Goddard et al., 1991; Pandolfi et al., 1991; Pandolfi et al., 1992), whilst bcr 2 breakpoints are associated with a 96-105 kD fusion protein (Pandolfi et al., 1992). There has been considerable interest to determine whether PML breakpoint pattern influences disease behaviour, and there is some data to suggest that this is indeed the case. Leukaemic blasts from a subgroup of patients with bcr 2 breakpoints have been found to
exhibit decreased sensitivity to ATRA in vitro in comparison with cases with bcr 1 and 3 breakpoints (Gallagher et al., 1995). Furthermore, a series of studies have suggested a relationship between the bcr 3 breakpoint and a variety of disease parameters including variant morphology (Biondi et al., 1992), elevated white cell count at presentation (Gallagher et al., 1997), presence of additional cytogenetic abnormalities (Slack et al., 1997) and an increased risk of relapse (Borrow et al., 1992; Vahdat et al., 1994). Subsequent studies have not confirmed the presence of a bcr 3 breakpoint as an independent adverse risk factor (Fukutani et al., 1995; Gallagher et al., 1997). These conflicting results most likely reflect variability in treatment approach within and between studies, compounded by relatively small sample size.

PML-RARα fusion products are subject to considerable heterogeneity generated not only by 3 possible PML breakpoints, but also due to alternative splicing involving exons 3,4,5 and 6 of PML in addition to the use of 2 alternative RARα polyadenylation sites (Pandolfi et al., 1992). PML exons 3-5 encode a predicted α helix, the most N-terminal part of which forms the coiled-coil domain, whilst exon 6 corresponds to part of the C-terminal serine/proline rich potential phosphorylation site and also includes a putative nuclear localisation signal (Fagioli et al., 1992; Kastner et al., 1992; Flenghi et al., 1995), see Figure 1.2. Whilst these components are variably retained in PML-RARα fusion products associated with bcr 1 and bcr 2 PML breakpoint patterns, bcr 3 breakpoints entail loss of the C-terminal phosphorylation site from PML-RARα associated with some reduction in α helix length; indeed products in which exon 3 is spliced out lack the coiled-coil domain altogether (Pandolfi et al., 1992). The alternative splicing of exons 3-6 noted in patients with 3' PML breakpoints is also present in the reciprocal RARα-PML transcripts of patients with 5' (bcr 3) breakpoints (Borrow et al., 1992; Alcalay et al., 1992).

This heterogeneity in fusion products generated by the t(15;17) creates confusion as to their relative roles in mediating leukaemogenesis and raises concerns regarding the selection of appropriate constructs for functional studies. However, since the majority of PML-RARα fusion products retain the N-terminal proline-rich region and RBCC domain there may be little functional difference between them with regard the ability to cause leukaemic transformation. More subtle differences between fusion products, for example altered length of α helix or coiled-coil motifs, the variable retention of phosphorylation domains and PML nuclear localisation signal and whether there is concomitant expression of RARα-PML could influence behaviour of the leukaemic
clone and hence account for correlations between PML breakpoint pattern and disease characteristics, should these be ultimately confirmed.

1.3 Role of RARα and retinoid signalling pathways in normal haemopoiesis

In order to understand the significance of RARα disruption in APL it is important to consider the normal mechanisms mediating retinoid signalling within the cell and their functional role. For many years it has been appreciated that retinoids exert profound effects on morphogenesis and differentiation suggesting an important role in embryogenesis (Brockes, 1990), which has subsequently been confirmed by transgenic approaches (Kastner et al., 1994; Sucov et al., 1994; Saitou et al., 1995). Furthermore, retinoids can achieve significant differentiating effects in a wide variety of tumour cell types, including teratocarcinomas and APL. Retinoid activity is mediated by two distinct families of nuclear receptors; the retinoic acid receptors (RARs : RARα, RARβ, RARγ) which can bind both ATRA and 9-cis RA, and the retinoid-X receptors (RXRs : RXRα, RXRβ, RXRγ) for which 9-cis RA is the only high affinity ligand (Leid et al., 1992a). The retinoid receptors may exist as monomers, homodimers or RAR/RXR heterodimers (Leid et al., 1992a), conferring their activity by binding to specific DNA response elements leading to activation or repression of target genes dependent on the presence or absence of ligand (reviewed by Beato, 1989; Leid et al., 1992a; Stunnenberg, 1993).

Steroid hormone receptors may be divided into two broad groups on the basis of their preferred dimerisation partner, DNA binding characteristics and features of the DNA binding motif (Beato, 1989; Stunnenberg, 1993). Hormone receptor response elements are composed of a hexanucleotide motif that may be repeated in a direct, inverted or palindromic pattern, separated by a variable number of spacer nucleotides (Beato, 1989; Leid et al., 1992a; Stunnenberg, 1993; Chambon, 1996). Analysis of naturally occurring and synthetic response elements linked to reporter genes suggest that type I receptors, such as glucocorticoid, progesterone and oestrogen receptors, bind preferentially to palindromic motifs separated by 3 nucleotides. Hence such receptors are believed to bind DNA as homodimers in a "head to head" fashion with dimerisation occurring at the C domains (Stunnenberg, 1993). In contrast, type II receptors, such as thyroid hormone (TR), vitamin D (VDR) and the RARs, require the presence of RXR for high affinity DNA binding (Zhang et al., 1992; Kliewer et al., 1992; Leid et al., 1992a,b; Stunnenberg, 1993). RXR/type II receptor heterodimers bind preferentially to response elements which form an inverted or direct repeat pattern favouring a "head to tail"
binding model (Luisi et al., 1991; Schwabe et al., 1993; Stunnenberg, 1993). The specificity of the DNA interaction is conferred by differences in the sequence and orientation of the hexanucleotide motif and also by variation in the number of spacer nucleotides (Umesono et al., 1991; Leid et al., 1992a; Stunnenberg, 1993). Hence, VDR binds preferentially to a DR+3 motif (direct repeat with 3 spacer nucleotides), TR to a DR+4 element and RAR typically, but not exclusively to DR+1, DR+2 or DR+5 motifs. In contrast, RXR homodimers bind preferentially to DR+1 elements (Umesono et al., 1991; Stunnenberg, 1993; Kurokawa et al., 1994). Although RXR binds to a range of response elements in partnership with RAR, there is evidence to suggest that RXR ligand responses are not mediated through such a complex; indeed, the formation of RAR/RXR heterodimers in some instances renders the interaction of RXR with its ligand unfavourable (Kurokawa et al., 1994; Forman et al., 1995). However, there is also data to suggest that in some reporter systems that the presence of both ligands is associated with a synergistic activation response (Durand et al., 1992: 1994). RXR responses appear to be mediated through RXR homodimers acting at DR+1 response elements (Kurokawa et al., 1994; Forman et al., 1995) and possibly through heterodimerisation with the orphan receptor NGFI-B at NGFI-B response elements (Forman et al., 1995). Ligand-bound RXR homodimers lead to transcriptional activation at DR+1 sites, in contrast to RAR/RXR heterodimers that bind such elements with greater affinity but have a repressive effect (Kurokawa et al., 1994). Further studies have revealed that the orientation of RAR/RXR heterodimers at DNA binding sites is a critical determinant of response, influencing release of the nuclear corepressor N-CoR (discussed below) from RAR in the presence of ligand (Kurokawa et al., 1994, 1995; Hörlein et al., 1995). At DR+1 elements RAR binds the motif upstream of RXR, associated with transcriptional repression with persistent N-CoR binding despite the presence of ligand binding to RAR. Whereas at DR+2 and DR+5 elements, RXR is the upstream partner and RAR ligand binding leads to N-CoR release and transcriptional activation (Kurokawa et al., 1993; 1994; Forman et al., 1995; Zechel et al., 1994a,b). Biochemical and structural studies to date have suggested that the polarity of heterodimer binding is determined by the specificity of the interaction between the components of the heterodimer and the response elements and steric hindrances (Rastinejad et al., 1995).

Review of the mechanisms of retinoid induced signalling reveals multiplicity at all levels of the pathway which may be essential to achieve their wide ranging and far-reaching effects (Leid et al., 1992a). Firstly, there is variability in ligand preferences for activation of a particular retinoid pathway. Both ATRA and 9-cis RA can activate
RARs, whereas only 9-cis RA can bind to RXRs, raising the possibility that a relative excess of a particular retinoid might lead to changes in response element binding patterns. For example, a rise in 9-cis RA in the cell might favour increased RXR homodimer binding to DR+1 response elements, possibly triggering a different repertoire of responses than if RAR/RXR mediated DR+2 or DR+5 elements had been activated (Leid et al., 1992a; Schrader et al., 1993). Levels of retinoids within the cell may be modulated by retinoid binding proteins, whose activity in the adult and during embryogenesis may be temporally and spatially restricted (Leid et al., 1992a). Cellular retinol binding proteins (CRBPs) bind preferentially to retinol; whereas cellular retinoic acid binding proteins (CRABPs) bind ATRA rather than 9-cis RA (Leid et al., 1992a). The CRABPs contain a retinoic acid inducible promoter region and have been considered to contribute to resistance to long term ATRA therapy in APL (Warrell, 1993).

The RARs and RXRs exist in a series of subtypes which share significant sequence homology across species, but little similarity between subtypes within the same species (Leid et al., 1992a). They vary in their pattern of distribution; for example RARα is ubiquitously expressed, RARβ is expressed in a variety of epithelial cell types, whereas expression of RARγ appears to largely confined to the skin (Collins et al., 1990). These spatial differences may be important for pattern formation during embryogenesis (Leid et al., 1992a). The retinoid receptor subtypes may demonstrate further variability generated by usage of two alternative promoter sites, which vary in retinoic acid inducibility, as well as by alternative splicing amongst the 5' exons. This creates a series of isoforms with variable N-terminal A domains fused to common B to F regions (Leroy et al., 1991), yielding for example two major isoforms of RARα (α1 and α2) (Chambon, 1996). This is particularly interesting since the rearrangement in APL also preserves the B to F domain of RARα, substituting the A domain for PML sequence. The A domain of the RARs may confer tissue specificity and ligand independent transactivation function (Leroy et al., 1991; Leid et al., 1992a).

Further investigation into the mechanisms underlying the repression mediated by unliganded hormone receptors and transactivation induced by ligand binding, has revealed further levels of complexity. It is now clear that transcriptional silencing at retinoid response elements by unliganded receptor is mediated by repressor molecules designated N-CoR (nuclear receptor corepressor) (Kurokawa et al., 1995; Hörlein et al., 1995) and SMRT (silencing mediator for RAR and TR) (Don Chen & Evans, 1995), which bind in helix 1 of the ligand-binding domain (Chambon, 1996). Transcriptional
repression is mediated through the recruitment of other proteins including SIN3 and histone deacetylase (HDAC) (Hörlein et al., 1995; Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997), leading to histone deacetylation rendering chromatin inaccessible to transcriptional activators or basal transcription machinery. The presence of ligand has been shown in structural studies to induce marked conformational change in the receptor, including the corepressor (CoR box) domain (Bourguet et al., 1995; Renaud et al., 1995). These conformational changes are likely to account for the polarity dependent dissociation of the corepressor complex including HDAC from the receptor following ligand binding, and the concomitant binding of coactivator proteins which mediate AF-2 activity. Coactivators characterised to date include the RING finger protein TIF1α (Transcription Intermediary Factor) (Le Douarin et al., 1995, 1996), SRC-1 (Steroid receptor coactivator) (Onate et al., 1995), TIF 2 (Voegel et al., 1996) and ACTR (Chen et al., 1997). Recent studies have demonstrated that ACTR (Chen et al., 1997) and SRC-1 (Spencer et al., 1997) possess intrinsic histone acetyltransferase activity, thereby targeting acetylation to promoters rendering chromatin more accessible favouring transcriptional activation (Utley et al., 1998). ACTR has been shown to interact with the coactivator CBP (CREB-binding protein), thereby providing a direct link with the basal transcription machinery (Chen et al., 1997). Furthermore, SRC-1, ACTR and CBP can interact in a multisubunit complex with p/CAF which also possesses histone acetyltransferase activity (Bannister & Kouzrides, 1996; Ogryzko et al., 1996; Chen et al., 1997; Spencer et al., 1997). Whilst alterations in histone acetylation appear to be important in regulating activity at steroid hormone response elements, recent work suggests that additional mechanisms are involved which may also be mediated through influences on chromatin structure (Wong et al., 1998). A further level of complexity is provided by cross modulation observed between retinoid and API1 (jun/fos) signalling pathways, which provides a potential link between activity at retinoid response elements and signals transduced by membrane receptors through the growth factor/Ras/Raf/MAP kinase cascade, with important implications for the regulation of cellular growth and differentiation (Chambon, 1996).

The diversity at all levels of the retinoid pathway is believed to facilitate the wide range of influences retinoids have on development, differentiation and homeostasis (Leroy et al., 1991; Leid et al., 1992a; Chambon, 1996). In view of the complex regulation of this system, it is also apparent that expression of abnormal retinoid receptors created by chromosomal translocations associated with APL could have far reaching consequences, potentially altering the character and repertoire of retinoid responses due to altered binding specificity to response elements. In addition a wide range of cellular processes
could potentially be disrupted through sequestration of RXR, influences on coactivators and corepressors and interference with AP-1 signalling pathways.

Characterisation of the retinoid receptors has permitted more detailed investigation to determine whether they possess a physiological role in cellular function and tissue development. Whilst integrity of the retinoid signalling pathways is clearly essential for normal embryogenesis particularly for heart and skin formation (Kastner et al., 1994; Sucov et al., 1994; Saitou et al., 1995; Imakado et al., 1995), a number of studies have highlighted the importance of RARα postnatally for normal myeloid differentiation. In particular, embryonic stem cells lacking RARα through homologous recombination were found to fail to mature into granulocytes (Labrecque et al., 1998). Similarly, defective granulopoiesis was observed in PML "knock out" mice, which were subsequently shown to have impaired RARα mediated responses, as described in more detail in the following section (Wang et al., 1998b). Furthermore, transfer of C-terminally truncated RARα into mouse bone marrow cells or the FDCP pluripotential cell line, thus inhibiting the activity of wild type receptor, led to maturation arrest at the promyelocyte stage (Tsai & Collins, 1993). Identical results were obtained when mouse bone marrow cells were transduced with RARα cDNA using a retroviral vector, leading to overexpression of wild type receptor (Onodera et al., 1995). Relatively little is known as to the key targets of RARα mediating myeloid differentiation, although there is some evidence to suggest the cyclin-dependent kinase inhibitor p21WAF1/CIP1 may be involved (Liu et al., 1996a,b; Wang et al., 1998b). The demonstration that RARα plays an important role in normal granulopoiesis, clearly implied that disruption of retinoid pathways plays a critical part in the pathogenesis and subsequent phenotype of APL. However, it should be noted that in neither of the previously described experimental systems involving overexpression of mutant or wild-type receptor did mice ultimately develop APL. Therefore, whilst disruption of retinoid pathways mediated by aberrant chimaeric hormone receptors is likely to contribute to the differentiation block that characterises this form of AML, these data would imply that the fusion partner, e.g. PML, is likely to play a significant role in the processes underlying leukaemic transformation.

1.4 Function of PML, evidence for localisation within multiprotein nuclear bodies

PML is expressed in a wide range of tissues and haemopoietic cell lines as a bewildering array of isoforms that range in molecular weight from 48-98kD (Goddard et al., 1991;
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Fagioli et al., 1992; Borrow & Solomon, 1992; Terris et al., 1995; Flenghi et al., 1995). Isoform variability is generated by alternative splicing within the central exons (4, 5 & 6) in addition to the utilisation of a series of potential carboxy termini (exons 7, 8 & 9) (Goddard et al., 1991; Fagioli et al., 1992; Borrow & Solomon, 1992). The significance of this variability is unclear at present, particularly as all isoforms retain the cysteine-rich motifs and coiled-coil domains that are considered of functional importance as protein-protein interaction domains (Goddard et al., 1991; Fagioli et al., 1992). However, it is possible that alternative splicing between the central exons could influence the distribution of PML within the cell, by an effect on the nuclear localisation signal (Kastner et al., 1992; Flenghi et al., 1995) whilst C-terminal variability might affect its biological activity by inserting regions with potential phosphorylation sites (Fagioli et al., 1992).

The successful raising of antisera against PML provided a significant step forward in defining its cellular function. Immunofluorescence staining revealed localisation predominantly within the nucleus in a speckled pattern corresponding to discrete multiprotein nuclear structures (0.3-0.5μm in diameter) known variably as PML nuclear bodies, ND10, Kr bodies or PODS (PML oncogenic domains) (Stuurman et al., 1992; Kastner et al., 1992; Daniel et al., 1993; Dyck et al., 1994; Weis et al., 1994; Borden et al., 1995). Determination of the function of these nuclear bodies has been a matter of considerable interest over the last few years: there are usually 10-30 per nucleus, they lie in a close relationship to chromatin, are distinct from nucleoli and spliceosomes (Weis et al., 1994) and do not associate with metaphase chromosomes (Flenghi et al., 1995).
Figure 1.3

Schematic representation of the components, potential functions and disease states targeting PML nuclear bodies. Nuclear body components associated with neoplasia: PML, PLZF, Rb, Int-6, RFP and CBP are denoted by a stippled background. Nuclear body components NDP 53, 55 and 65 have not been formally characterised. For full review of the constituents and proposed functions of PML nuclear bodies please refer to Hodges et al., (1998) from which this figure is adapted.
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Recent studies reveal them to be dynamic structures which could include more than 10 component proteins, a number of which were identified as targets of autoantibodies derived from patients with autoimmune diseases, such as primary biliary cirrhosis (Dyck et al., 1994; Weis et al., 1994; Hodges et al., 1998). Subsequently, PML nuclear bodies have been found to be associated with an extremely wide range of disease processes, thereby implying an important functional role, see Figure 1.3. Of particular interest was the discovery that these structures are disrupted in APL cases with the t(15;17) raising the possibility that this process could play an important role in oncogenesis (Daniel et al., 1993; Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994).

Most recently, alteration of PML nuclear bodies has been implicated in the neurodegenerative condition Spinocerebellar ataxia type 1 (Skinner et al., 1997). This disorder is associated with mutations in the SCA1 gene encoding an aberrant ataxin-1 protein associated with an expanded polyglutamine tract. Wild-type ataxin-1 has been found to localise to PML nuclear bodies in cerebellar Purkinje cells, whilst expression of mutant ataxin-1 leads to disruption of the nuclear bodies and delocalisation of PML (Skinner et al., 1997). The PML nuclear bodies are also targeted by a wide range of viral infections, including herpes simplex (Everett & Maul, 1994), adenovirus (Puvion-Dutilleul et al., 1995; Carvalho et al., 1995; Doucas et al., 1996), cytomegalovirus (Kelly et al., 1995), Epstein-Barr virus (EBV) (Szekely et al., 1996) and HTLV1 (Desbois et al., 1996). In EBV infection, the EBNA-5 protein has been found to colocalise with PML within apparently intact nuclear bodies, whilst the other infections lead to redistribution of their constituents. For example, in HTLV1 infection which is associated with adult T cell leukaemia/lymphoma, the viral Tax oncoprotein causes relocation of Int-6 from the nuclear bodies to the cytoplasm, but without altering the distribution of PML. This phenomenon could play a role in tumourigenesis, since Int-6 has been shown to be converted into a dominant negative oncogene after retroviral insertion (Marchetti et al., 1995). In other infections PML itself is redistributed. In the case of herpes simplex (HSV) infection, the immediate early viral protein Vmw110 (ICP0) which is required for reactivation of latent virus and also incidentally contains a RING finger domain, colocalises with PML nuclear bodies early in the course of infection (Everett & Maul, 1994). With subsequent progression, PML and Vmw110 are ultimately translocated together to the cytoplasm (Everett & Maul, 1994). These findings prompted the suggestion that PML nuclear bodies could mediate a storage function, harbouring components critical to the progression of viral infection. Furthermore, it was suggested that PML nuclear bodies could be involved in transport
between the nucleus and cytoplasm (Everett & Maul, 1994). Interestingly in this regard, PML has been shown to interact with a ubiquitin homology domain protein PIC1 (also known as UBL1, SUMO1, GMP1) (Boddy et al., 1996; Müller et al., 1998). PIC1 has itself been shown to modulate the subcellular localisation of the Ran GTPase-activating protein (RanGAP), a component of the nuclear import apparatus, targeting it to the nuclear pore complex (Matunis et al., 1996; Mahajan et al., 1997). PIC1, which is believed to be conjugated to PML in a phosphorylation-dependent manner involving the human form of the ubiquitin conjugating enzyme Ubc9, has also been proposed to play an important role in targeting PML to the nuclear bodies (Müller et al., 1998). Therefore, modulation of these interactions could underly the alteration of nuclear bodies associated with a number of disease states. Interestingly, recent studies have shown that the HSV protein ICPO (Vmw110) can interact with the nuclear body constituent HAUSP (Herpes virus Associated Ubiquitin Specific Protease) (Everett et al., 1997). More prominent HAUSP staining has been observed in nuclear bodies following viral infection suggesting that ICPO can recruit HAUSP, which could potentially disrupt the PML/PIC1 interaction through protease activity thereby accounting for the subsequent delocalisation of PML associated with HSV infection (Müller et al., 1998). This redistribution of PML in viral infections is interesting, in the context of studies demonstrating marked expression of PML in macrophages, particularly associated with various inflammatory and neoplastic processes (Flenghi et al., 1995; Terris et al., 1995). PML expression appears to be upregulated in neoplastic cells of a wide range of tumours, in cells affected by various inflammatory processes, in normal tissues associated with proliferation and in promonocytic cells induced to differentiate with combinations of vitamin D3 and transforming growth factor β1 with or without interferon-γ (Flenghi et al., 1995; Terris et al., 1995). Whether these findings are of any functional significance, perhaps indicating that PML is a marker of the proliferative state of the cell and consistent with a role in cell growth, or merely reflect a non-specific cytokine mediated effect is not entirely clear at present. Nevertheless, it is now apparent that a number of nuclear body proteins are upregulated by interferons (Chelbi-Alix et al., 1995). More detailed investigation of PML has revealed that, at least in this case, the phenomenon is mediated through specific response elements contained within the promoter region (Stadler et al., 1995). This raised the possibility that up-regulation of nuclear body constituents could contribute to the host defence against viral infection and suggested that antiviral effects of interferons could be mediated by this mechanism (Pellicano et al., 1997; Chelbi-Alix et al., 1998). Further support for a role of PML in defence against infection has been derived from studies of "knock-out" mice, which were found to succumb to severe infections (Wang et al., 1998b); however, it
should be noted that granulopoiesis was defective in these mice which could have accounted for this phenomenon.

A number of lines of evidence have suggested that PML functions as a growth suppressor, raising the possibility that disruption of this gene could play an important role in leukaemogenesis (Mu et al., 1994; Liu et al., 1995). Transfection of PML into APL-derived NB4 cells which carry the PML/RARα rearrangement suppressed their anchorage-independent growth on soft agar and tumourigenicity in nude mice. PML also suppressed transformation of rat embryo fibroblasts by Ha-ras in the presence of mutant p53 or c-myc. Furthermore PML prevented the transformation of NIH3T3 cells by activated neu oncogene, an effect that was abrogated by co-transfection with PML-RARα. Function of PML as a growth suppressor has been subsequently supported by the growth characteristics of fibroblasts derived from PML "knock out" mice, in comparison to cells derived from wild type controls (Wang et al., 1998b). Furthermore, "knock-out" mice were found to have an increased susceptibility to development of tumours induced by carcinogens, although the premature death of mice due to infections did not permit determination of the longer term consequences of PML disruption.

Interestingly, two further members of the family of RING finger proteins characterised by the RBCC tripartite motif, namely RFP (Ret Finger Protein) and steroid hormone coactivator TIF 1, have also been found to be oncogenic in the context of chimaeric fusion proteins (Takahashi et al., 1988; Miki et al., 1991; Le Douarin et al., 1995; Kastner et al., 1992; Freemont, 1993). Moreover, RFP has been shown to interact with PML and colocalise within the nuclear bodies (Cao et al., 1998). This raises the possibilities that deregulation of pathways involving proteins interacting with the various domains of the tripartite motif and disturbance of the dynamics of the nuclear bodies themselves could play important roles in oncogenesis. Studies have already identified a number of interaction partners of PML which could be involved in this process. The first such protein to be identified, using the yeast two-hybrid system, was PIC1 (for Pml Interacting Clone) (Boddy et al., 1996) which has since been implicated in cellular localisation of PML as mentioned above (Kamitani et al., 1998; Müller et al., 1998). PIC1 was found to possess homology with the S. cerevesiae protein SMT 3 (Boddy et al., 1996), which was identified as a suppressor of mutations in MIF2 (Meluh & Koshland, 1995a,b). MIF2 has been shown to localise to the core centromere (Meluh & Koshland, 1995b) and is required for mitotic spindle integrity during anaphase (Brown et al., 1993). PIC1 has also been found to interact with RAD 51 and RAD 52 suggesting a potential role in DNA repair (Shen et al., 1996). Subsequent studies have
also suggested that the transcriptional repressor PLZF (for Promyelocytic Leukaemia Zinc Finger) also interacts with PML (Koken et al., 1997). This is of particular interest as PLZF is also directly implicated in APL through its disruption by the reciprocal translocation t(11;17)(q23;q21) (Chen et al., 1993a,b) which is described in more detail in section 1.5. Furthermore, a recent study has demonstrated that the hypophosphorylated fraction of the retinoblastoma protein (pRB) localises to the nuclear bodies through an interaction with PML (Alcalay et al., 1998). This appears to involve the B-box domain and carboxy terminus of PML and the pocket region of RB; although further investigations suggested that the growth suppressor effects of PML are not mediated through pRB. However, PML was found to abolish the activation of glucocorticoid receptor by pRB, leading to the suggestion that PML could form a component of a transcription regulatory complex (Alcalay et al., 1998). This hypothesis is supported by the finding that mice lacking PML through homologous recombination have a defect in terminal myeloid differentiation, reflecting disruption of retinoid signalling pathways (Wang et al., 1998b). Further studies revealed that PML was required for retinoic acid dependent transactivation of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 gene, which regulates cell cycle progression and is involved in the control of cellular differentiation (Wang et al., 1998b). The precise mechanisms underlying this effect remain undetermined at present, but could reflect an interaction with steroid hormone coactivators, corepressors or a more general effect on chromatin structure or the basal transcription machinery. However, a recent study has detected the steroid hormone coactivator CREB-binding protein (CBP) (Chakravarti et al., 1996) within the nuclear bodies, suggesting a close relationship between PML and the basal transcription machinery (LaMorte et al., 1998). Indeed, in vivo nucleic acid labelling revealed nascent RNA polymerase II transcripts within the nuclear body further supporting a role for components of these structures in transcriptional regulation (LaMorte et al., 1998). The presence of CBP in the nuclear bodies is of interest due to its wide range of interaction partners including the cyclic-AMP response element binding protein (CREB)(Chrivita et al., 1993; Kwok et al., 1994), AP 1 (jun/fos)(Arias et al., 1994), and the viral oncoproteins, E1A (Arias et al., 1994; Lundblad et al., 1995 Arany et al., 1995) and Tax (Kwok et al., 1996) which are derived from adenovirus and HTLV 1, respectively. Furthermore, CBP itself is implicated in oncogenesis through its involvement in the Rubinstein-Taybi syndrome (Petrij et al., 1995). More recently CBP has been associated with haematological malignancy through its disruption by the t(8;16)(p11;p13) rearrangement in AML M4/M5, leading to the formation of a fusion gene involving MOZ, which encodes a zinc finger protein with putative histone acetyltransferase activity (Borrow et al., 1996); or by the t(11;16)(q23;p13) involving
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the promiscuous fusion partner MLL, leading to myelodysplasia (Taki et al., 1997). Further evidence for a role of the nuclear bodies in regulation of gene expression was provided by a recent report demonstrating that the nuclear body constituent SP100 interacts with heterochromatin protein 1 (HP 1), an interaction partner of TIF 1 (Le Douarin et al., 1996) which is believed to be an important structural component of heterochromatin. Furthermore, a splice variant of SP100 (SP100-HMG) was identified with homology to the high mobility group-1 protein family, which in common with SP-100 and HP 1 was found to exhibit transcriptional repression activity (Seeler et al., 1998).

As to whether the transcriptional regulatory and growth suppressor effects attributed to PML are mediated by the protein itself, and if so whether these functions are dependent on the presence of intact nuclear bodies, or indeed whether they are mediated through other nuclear body components independent of PML, remains to be established. Further information regarding the function of PML and the nuclear bodies is likely to be derived from additional studies identifying proteins interacting with the tripartite motif of PML and factors determining the integrity of the nuclear bodies. Determination of the solution structure of the RING motif of PML by NMR, permitted very specific site-directed mutagenesis studies exploring the key domains required for incorporation into nuclear bodies (Borden et al., 1995). Mutations of cysteines involved in either the first or second zinc binding site prevented localisation of PML to nuclear bodies, whilst mutations involving surface amino acids far from the zinc binding sites had no such effect (Borden et al., 1995). This study lent weight to the view that the RING finger domain is important for mediating protein-protein interactions which may be essential for the integrity of the nuclear bodies. Similarly point mutations within either B box domain prevented PML localisation within nuclear bodies; this suggested that correct targeting is dependent on interaction with other factors (possibly PICl), since none of the above mutations disrupted PML homodimerisation which is mediated by the coiled-coil domain (Borden et al., 1996). As to whether PML is a critical structural component of the nuclear bodies remains to be determined, since lack of availability of suitable antisera has prevented determination as to whether other nuclear body constituents still coalesce in mice lacking PML (Wang et al., 1998b).

Interestingly, a significant number of nuclear body proteins are directly implicated in oncogenesis, including Int-6, RFP, RB, PML, PLZF and CBP. This implies that the nuclear body plays an important role in control of cell growth and suggests that deregulation of nuclear body constituents could provide an important common pathway.
to the development of neoplasia. It is becoming clear that characterising the role of the nuclear bodies, their composition, the determinants of their stability and their relationship with PML is fundamental to understanding the pathogenesis of APL, and its response to ATRA and novel agents such as arsenic containing compounds. The recent description of rare cases of APL with alternative translocations which do not disrupt PML, provide a further opportunity to explore the role of PML and the nuclear bodies in APL pathogenesis in general.

1.5 Alternative translocations associated with APL

Early cytogenetic studies performed in specialised centres had initially suggested that the t(15;17) is detected in all cases of morphologic APL (Larson et al., 1984). However, it is now clear that in rare instances morphological APL is associated with distinct alternative balanced translocations which also involve RARα. These include the t(11;17)(q23;q21) (Chen et al., 1993a,b), t(11;17)(q13;q21) (Wells et al., 1997) and t(5;17)(q32;q12) (Redner et al., 1996) abnormalities, whereby RARα is fused to PLZF (Promyelocytic Leukaemia Zinc Finger), NuMA (Nuclear Mitotic Apparatus protein) or NPM (NucleoPhosMin) genes, respectively in place of PML. Interestingly, in common with the disease associated with the classic t(15;17), in each case RARα was found to be disrupted within its second intron thereby preserving DNA-, coactivator/-corepressor-, RXR- and ligand-binding domains within the respective fusion proteins, see Figure 1.4. This is consistent with a role for retinoid receptors in normal myelopoiesis and suggests that the chimaeric receptors deregulate this process leading to the differentiation block that characterises APL. Although such alternative translocations are extremely rare, accounting for less than 1% of morphologic APL (Pandolfi, 1996), elucidation of the mechanisms underlying leukaemogenesis in these cases is likely to provide considerable insight into the processes involved in the development of PML-RARα mediated disease, including the role of PML nuclear bodies in the pathogenesis of APL. In particular, cases with alternative translocations afford the opportunity to dissect out mechanisms leading to leukaemic transformation from those mediating the block in myeloid differentiation and its reversal by retinoids.
Figure 1.4

Chimaeric fusion products arising from alternative APL associated translocations. Each of the chromosomal rearrangements disrupts RARα within the 2nd intron, leading to retention of the hormone receptor DNA-, RXR-, ligand-, coactivator- and corepressor-binding domains within the fusion protein.
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1.5.1 APL associated with the PLZF/RARα rearrangement

The t(11;17)(q23;q21) abnormality associated with a PLZF/RARα rearrangement is the commonest of the alternative translocations with 8 cases reported to date (Chen et al., 1993a,b; Licht et al., 1995; Jansen et al., 1996; Culligan et al., 1998). Morphological appearances differ somewhat from APL with the classic t(15;17); in particular, cases may fall between FAB subtypes M2 and M3, display dysplastic features, whilst Auer rods tend to be sparse (Licht et al., 1995). Furthermore, in marked contrast to t(15;17) associated APL, blasts from PLZF/RARα cases have been found to be resistant to the differentiating effects of retinoids, such that treatment of patients with ATRA as single agent therapy fails to induce clinical remission accounting for the high induction death rate (Licht et al., 1995). These findings led to the suggestion that t(11;17) APL is a distinct clinico-pathological entity associated with an adverse prognosis (Licht et al., 1995); however a more recent report has demonstrated that complete remission is achievable in this condition using induction therapy incorporating combination chemotherapy (Culligan et al., 1998). Clearly, these studies serve to highlight the importance of molecular and cytogenetic characterisation of all newly diagnosed cases of APL as a means of determining the optimum treatment approach.

PLZF, which is believed to function as a transcriptional repressor, is structurally unrelated to PML, being a member of the POK (POZ and Krüppel) family of proteins, defined by an N-terminal POZ domain and C-terminal Krüppel-like C2-H2 zinc fingers which mediate DNA binding (Chen et al., 1993b; Li et al., 1997b). PLZF contains 9 zinc fingers; in the majority of cases characterised to date the t(11;17) leads to disruption within the intron separating the exons encoding the second and third zinc fingers; whilst in a single case a more 3' breakpoint in PLZF was detected leading to the retention of 3 Krüppel-like zinc fingers in the PLZF-RARα fusion. The reciprocal RARα-PLZF fusion product retains the remaining C-terminal zinc fingers and has been shown to be capable of specific DNA binding (Sitterlin et al., 1997; Li et al., 1997b), suggesting that it may also play an important contributory role in leukaemogenesis (Licht et al., 1995; Sitterlin et al., 1997; Li et al., 1997b). This contrasts with t(15;17) associated APL in which the role of reciprocal derived RARα-PML is more uncertain.

A number of C2-H2 zinc finger transcription factors have been shown to play an important role in haemopoiesis including MZF-1 (Bavisotto et al., 1991), Ikaros (Georgopoulos et al., 1992: 1994) and EKLF (Miller & Bieker, 1993; Nuez et al., 1995; Perkins et al., 1995), suggesting that PLZF may also be involved in this system. In
acCORDANCE WITH THIS HYPOTHESIS PLZF HAS BEEN FOUND TO BE EXPRESSED AT HIGHEST LEVELS IN UNDIFFERENTIATED, MULTIPOTENTIAL HEMATOPOIETIC PROGENITOR CELLS, WHILST EXPRESSION LEVELS DECLINE WITH LINEAGE COMMITMENT AND DIFFERENTIATION (REID ET AL., 1995). THIS RAISES THE POSSIBILITY THAT PLZF MAY PLAY A ROLE IN MAINTENANCE OF THE PHENOTYPE OF UNCOMMITTED HEMATOPOIETIC PROGENITORS AND/OR PERHAPS INFLUENCE LINEAGE COMMITMENT. Whilst no common structural motifs appear to link PML and PLZF, thereby accounting for their leukemic potential in the context of formation of fusion proteins with RARα, subsequent studies have revealed that these proteins share a number of common features. In particular, PLZF is also localised to discrete nuclear bodies (reid et al., 1995; leicht et al., Licht et al., 1996; Ruthardt et al., 1998), whose formation is dependent on the integrity of the POZ domain (dong et al., 1996) and in addition has been found to exhibit growth suppressor activity (pandolfi, 1996; shaknovich et al., 1998). The cyclin A2 promoter has been identified as a potential target for PLZF, which may be pertinent to the latter phenomenon (li et al., 1997b). Furthermore, recent studies have suggested that there is at least partial colocalisation of PML and PLZF within the nucleus reflecting a direct interaction between the two proteins (koken et al., 1997). This raises the possibilities that their growth suppressor activities might be interrelated and that fusion proteins associated with alternative APL translocations could promote leukemogenesis by a common pathway involving disruption of PML nuclear bodies and/or deregulation of their components.

1.5.2 APL associated with rearrangements of NuMA and NPM

The two remaining alternative translocations are extremely rare with only single cases reported to date. The t(5;17) which leads to formation of NPM-RARα and RARα-NPM fusion genes was identified in a case of paediatric APL (Redner et al., 1996). NPM is a highly conserved nucleolar phosphoprotein which functions as an oligomer (liu & Chan, 1991). It is thought to be involved with RNA processing, in particular transporting ribosomal ribonucleoproteins between the nucleolus and the cytoplasm during ribosomal assembly (Schmidt-Zachmann et al., 1987; Borer et al., 1989; Hernandez-Verdun, 1991). NPM expression has been found to be cell cycle regulated, with highest levels observed immediately prior to S-phase (Peter et al., 1990; Feuerstein, 1991; Feuerstein & Randazzo, 1991). NPM has been found to bind to double-stranded DNA and copurify with the DNA polymerase α-primase enzyme complex, suggesting a role in DNA replication (Takemura et al., 1994). In addition there is some evidence to suggest that NPM could modulate the activity of transcription factors such as YY1, potentially
affecting *fos* expression (Shi *et al.*, 1991; Seto *et al.*, 1993; Inouye & Seto, 1994) which could itself influence retinoid signalling pathways. Furthermore, recent data suggests that NPM, in common with PML and PLZF may also function as a growth suppressor (Pandolfi, 1996), thereby potentially providing a common link for the transformation potential of their respective fusion proteins. Interestingly, NPM is disrupted by two other reciprocal translocations which are associated with different forms of haematological malignancy. The t(2;5)(p23;q35), which is associated with Ki-1 positive anaplastic large cell lymphoma, leads to the fusion of the amino terminal region of NPM including a potential protein kinase C phosphorylation site and metal binding motif to a tyrosine kinase catalytic domain of a novel gene, *ALK* (Morris *et al.*, 1994). Whereas the t(3;5)(q25.1;q34) translocation associated with myelodysplastic syndrome and AML leads to the formation of a fusion involving a novel gene named *MLF1* (Yoneda-Kato *et al.*, 1996). This translocation has been described in all subtypes of AML with the notable exception of M3 (Raimondi *et al.*, 1989). The resultant fusion includes an additional 58 amino acids of NPM in comparison to NPM-ALK and NPM-RARα which involve identical breakpoints within NPM (Yoneda-Kato *et al.*, 1996; Morris *et al.*, 1994; Redner *et al.*, 1996), thereby implying that domains within the amino-terminus of this protein are important for oncogenesis.

Recently the breakpoint region of the t(11;17)(q13;q23) identified in a paediatric case of APL was cloned, revealing disruption of the gene encoding NuMA (Wells *et al.*, 1996; 1997). This large (approx 240kD), abundant coiled-coil protein is believed to play an important role in spindle function during mitosis and nuclear reassembly during telophase (Compton *et al.*, 1992; Yang *et al.*, 1992; Compton & Cleveland, 1994; Cleveland, 1995). Furthermore there is evidence to suggest that NuMA plays a role in the interphase nucleus as an important component of the nuclear matrix (Cleveland, 1995) and may be involved in RNA processing (Zeng *et al.*, 1994). The t(11;17)(q13;q23) abnormality was found to lead to the fusion of the amino-terminal 1883 amino acids of NuMA, including the globular domain which is involved in nuclear reassembly and the α-helical oligomerisation domain to the B-F domains of RARα (Wells *et al.*, 1997). No evidence was found for expression of reciprocal RARα-NuMA fusion transcripts.

That no cases of APL have been identified in which *PML, PLZF, NuMA* or *NPM* are fused to a novel gene suggest that disruption of these genes is not critical to the development of the promyelocytic phenotype, but may be essential for the process of leukaemic transformation. In this regard, growth suppressor activity has been
demonstrated for at least three of the fusion partners. Also, all of them appear to function as oligomers and/or heterodimers, raising the possibilities that this phenomenon could contribute to leukaemogenesis through sequestration of factors required for retinoid signalling and/or through an interaction with a common factor, such as PLZF or indeed another constituent of the PML nuclear body.

Whilst the number of cases of APL with alternative translocations described to date is extremely small, results of in vitro differentiation assays and clinical data, respectively suggest that AML associated with NPM/RARα and NuMA/RARα rearrangements is retinoid responsive (Redner et al., 1997; Wells et al., 1997) in accordance with the PML-RARα fusion, but in marked contrast to the retinoid resistance associated with the PLZF/RARα rearrangement. Whilst it appears likely that the amino-terminal portion of the various fusion partners in the chimaeric receptor protein share important roles in the pathogenesis of APL; in particular underlying leukaemic transformation and disruption of retinoid signalling pathways through altered binding to retinoid response elements, compounded by sequestration of RXR and coactivators/corepressors mediated by the RARα moiety, it also seemed possible that the nature of the fusion partner could influence the differentiation response to retinoids. Recent studies have addressed this important issue, delineating the molecular mechanisms underlying the differential response to retinoids of t(15;17) and t(11;17)(q23;q21) associated APL, thereby yielding significant advances in understanding the pathogenesis of this disorder, which are described below.

1.6 Molecular pathogenesis of APL and the response to ATRA

Leukaemia has traditionally been considered to arise out of a multistep process involving a number of accumulated genetic changes leading to defects in both growth and differentiation (Sawyers et al., 1991). However, the demonstration that PML, PLZF and NPM possess growth suppressor activity (Mu et al., 1994; Koken et al., 1995; Pandolfi, 1996; Shaknovich et al., 1998) and that expression of mutant RARα molecules leads to maturation arrest at the promyelocyte stage of myeloid differentiation (Tsai & Collins, 1993; Onodera et al., 1995) suggested that the fusion genes arising from the APL associated translocations could be sufficient in themselves to mediate leukaemogenesis. Indeed, subsequent studies generating transgenic mice expressing PML-RARα or PLZF-RARα have confirmed the leukaemogenic potential of these gene fusions (Brown et al., 1997; Grisolano et al., 1997, He et al., 1997; 1998).
With the development of PML antisera it became apparent that expression of the PML-RARα fusion protein leads to significant alteration in nuclear architecture with disruption of PML nuclear bodies (Dyck et al., 1994; Weis et al., 1994; Koken et al., 1994). In cells derived from normal and neoplastic tissues or from haematological malignancies other than APL, less than 30 such nuclear bodies are typically observed, whilst in APL cases with the t(15;17) a highly characteristic microspeckled distribution of PML is detected (Dyck et al., 1994; Weis et al., 1994). Treatment of APL cells with ATRA leads to normalisation of the PML nuclear staining pattern as differentiation occurs (Dyck et al., 1994; Weis et al., 1994; Koken et al., 1994). Similarly, reversion to a wild-type pattern of PML staining has been observed when APL blasts are exposed to arsenic compounds, which induce remission through apoptosis rather than differentiation (Zhu et al., 1997). It has however been noted that some cases of APL with PML/RARα rearrangements are resistant to retinoids and maintain a microparticulate distribution of PML (Dyck et al., 1994). This prompted the suggestion that disruption of PML bodies plays a fundamental role in the pathogenesis of APL and that reversal of this process is essential to overcoming the maturation arrest that characterises the disease (Dyck et al., 1994).

A number of studies have also suggested that sequestration of RXR by the various APL associated fusion proteins could contribute to the differentiation block. Sequestration could occur through binding of RXR to the E domain of the retained carboxy-terminal portion of RARα, compounded by oligomerisation of the fusion protein mediated through the amino terminus of PML, PLZF, NuMA or NPM respectively (Weis et al., 1994; Jansen et al., 1995; Pandolfi, 1996; Wells et al., 1997). Accordingly, immunofluorescence studies performed in APL blasts demonstrated colocalisation of PML-RARα and RXR within microspeckled "structures", whilst ATRA treatment led to reversion to the typical nuclear diffuse localisation pattern of RXR (Weis et al., 1994). Furthermore, vitamin D3 dependent differentiation of the promonocyte cell line U937 was found to be blocked in cells expressing PML-RARα; again consistent with sequestration of RXR leading to impairment of RXR/VDR dependent transactivation (Grignani et al., 1993; Testa et al., 1994). In agreement with results obtained in APL blasts, the differentiation block was overcome by treatment with ATRA.

More recent studies have revealed that PML nuclear bodies contain components potentially influencing chromatin structure and have suggested that they play an important role in regulation of gene expression (discussed in section 1.4). Apart from the
APL associated proteins PML and PLZF which both possess growth suppressor activity, the nuclear bodies include a number of factors directly implicated in tumourigenesis, including CBP, RB, Int-6 & RFP (see section 1.4) suggesting that disruption of these structures could play a significant role in deregulation of cellular growth and contribute to leukaemic transformation. Central, to the understanding of this issue is clarification of the factors determining the structural integrity of the nuclear bodies and how these are influenced by the respective APL fusion proteins. Recently progress has been made in establishing the mechanisms underlying nuclear body disruption by the PML-RARα fusion protein and their subsequent reconstitution following therapy (Grignani et al., 1996). Extensive studies using haemopoietic cell lines expressing a variety of PML-RARα mutants established that the second heptad repeat of the coiled-coil and amino-terminal regions of the PML portion are essential to delocalise wild-type PML from nuclear bodies, implying that this reflects an interaction between their respective coiled-coil domains which are involved in PML homodimer and PML-RARα/PML heterodimer formation (Perez et al., 1993; Borden et al., 1996). Interestingly however, these studies also established that PML-RARα mutants lacking RING and B-box motifs or the second heptad repeat of the coiled-coil, which were both functionally active, independently led to delocalisation of the nuclear body constituent SP100, without influencing PML distribution (Grignani et al., 1996). This experiment established that the differentiation block mediated by PML-RARα is not dependent upon redistribution of PML, at variance with the hypothesis proposed by Dyck et al. (1994); although this approach could not address whether PML delocalisation and nuclear body disruption play a fundamental role in leukaemic transformation. Interestingly, Grignani et al.'s studies did not support RXR sequestration as a key factor mediating the block in differentiation; whilst integrity of the coiled-coil of the PML component of the fusion protein was found to be essential for this effect. This study therefore again highlighted the important contribution of the fusion partner to the development of APL and raised the possibility that acquisition of a heterologous dimerisation interface by RARα could be a key factor underlying leukaemogenesis (Grignani et al., 1996). Recent studies have also shed some light on the processes underlying nuclear body reformation following ATRA and arsenic therapy. Despite their distinct modes of action, both classes of compound lead to degradation of PML-RARα (Raelson et al., 1996; Yoshida et al., 1996; Zhu et al., 1997; Müller et al., 1998); in the case ATRA therapy this has recently been reported to be mediated through the caspase pathway (Nervi et al., 1998). With both therapeutic agents, the time course of fusion protein degradation is closely correlated with nuclear body reconstitution, suggesting that these phenomena are linked (Müller et al., 1998). It has been claimed that the nuclear body constituent PIC1 is involved in targeting PML to the nuclear
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bodies (Müller et al., 1998). Should this hypothesis be confirmed, it is possible that reformation of the nuclear bodies is facilitated by release of sequestered PIC1 as the fusion protein is degraded. Interestingly, however a recent study found that the growth inhibitory and apoptosis inducing effects of arsenicals are not specific to APL and indeed are also observed in murine embryonic fibroblasts lacking PML through homologous recombination, indicating that the mode of action of these agents is not dependent upon PML or reconstitution of the nuclear bodies (Wang et al., 1998c).

There has been considerable interest over the last few years to determine the mechanisms underlying the differential response to ATRA of APL cases associated with the PLZF-RARα fusion which are retinoid resistant (Licht et al., 1995), contrasting starkly with PML-RARα associated disease which is sensitive (Miller et al., 1992). The latter phenomenon raised a further fundamental issue; requiring an explanation for the occurrence of a block in differentiation at physiological levels of ATRA, which can be overcome by pharmacological levels of retinoid. A number of early studies clearly established that PML-RARα and PLZF-RARα are capable of binding retinoid response elements with similar affinity to wild type receptors (Kakizuka et al., 1991; de Thé et al., 1991; Pandolfi et al., 1991; Kastner et al., 1992; Chen et al., 1994; Licht et al., 1996). This would suggest that the differentiation block in APL could reflect altered response element binding preferences of the fusion proteins compared to wild-type receptors, potentially compounded by competition for DNA binding sites and sequestration of RXR leading to an altered repertoire and character of retinoid responses. In accordance with this hypothesis, a number of studies noted a repressive effect of PML-RARα and PLZF-RARα at retinoid response elements at physiological levels of ATRA (Kakizuka et al., 1991; de Thé et al., 1991; Pandolfi et al., 1991; Kastner et al., 1992; Chen et al., 1994; Licht et al., 1996). Interestingly, treatment with ATRA converted PML-RARα into a transcriptional activator (Kakizuka et al., 1991; de Thé et al., 1991; Pandolfi et al., 1991; Kastner et al., 1992), whilst in one study PLZF-RARα was found to have a persistent repressive effect in the presence of ATRA (Chen et al., 1994). More recent studies have suggested that the recently discovered corepressor molecules (discussed in section 1.3) could hold the key to the differentiation block that characterises APL and also account for differences in behaviour between PML-RARα and PLZF-RARα associated disease in response to retinoids. This latter phenomenon appears to reflect differential binding of the fusion proteins to SMRT or N-CoR (He et al., 1998; Guidez et al., 1998; Lin et al., 1998; Grignani et al., 1998). At physiological levels of RA both fusion proteins bind the corepressor, leading to recruitment of SIN3 and histone deacetylase (HDAC) and repression at retinoid response elements.
Furthermore, disruption of this interaction through mutation of PML-RARα within the CoR box, abolished the ability of the fusion protein to block differentiation (Grignani *et al.*, 1998). In the presence of pharmacological levels of RA, binding of ligand to the PML-RARα and PLZF-RARα fusion proteins is associated with displacement of the corepressor complex from the retinoid receptor moiety (He *et al.*, 1998; Guidez *et al.*, 1998; Lin *et al.*, 1998; Grignani *et al.*, 1998). This implies there is a conformational change in the receptor induced by ligand, permitting coactivator binding (see section 1.3), which in the case of PML-RARα leads to transcriptional activation at retinoid response elements. However, PLZF-RARα additionally binds N-COR/SMRT through the amino-terminal part of PLZF. This latter process is not influenced by the presence of retinoid and hence at pharmacological levels of RA the corepressor complex remains bound to the PLZF moiety of the PLZF-RARα fusion protein preventing transcriptional activation, which could account for the lack of sensitivity of t(11;17) associated APL to ATRA (He *et al.*, 1998; Guidez *et al.*, 1998; Lin *et al.*, 1998; Grignani *et al.*, 1998). Similarly leukaemic blasts derived from mice expressing PLZF-RARα were found to be resistant to conventional doses of ATRA (He *et al.*, 1998). Interestingly, however, differentiation was successfully induced using ATRA in combination with the deacetylating agent trichostatin A (TSA), but not with TSA alone (He *et al.*, 1998). As to whether this approach could prove successful in patients with t(11;17) associated APL (e.g. using ATRA in combination with sodium butyrate) is currently uncertain; since it remains a possibility that RARα-PLZF which is upregulated by ATRA could contribute to retinoid resistance due to persistent deregulation of the cell cycle (Licht *et al.*, 1995; Sitterlin *et al.*, 1997; Li *et al.*, 1997b).

Therefore, it appears likely that repression of retinoid responsive genes is likely to underly the differentiation block associated with all molecular subtypes of APL. Studies employing haemopoietic cell lines stably expressing mutant forms of PML-RARα, indicate that the fusion partner is essential to mediate this effect (Grignani *et al.*, 1996); this could reflect structural constraints on the fusion product influencing its binding preferences for various retinoid response elements, and/or interactions with other intermediates through dimerisation interfaces. Clearly, the nature of the fusion partner also has a significant bearing on the retinoid responsiveness of the leukaemia through a capacity to bind corepressor molecules. This would imply that in common with PML, amino-terminal regions of NPM and NuMA do not interact with SMRT or N-CoR, since APL cases involving the latter genes appear to be retinoid responsive (Redner *et al.*, 1997; Wells *et al.*, 1997). Interestingly, LAZ3/BCL6 which is involved in non Hodgkin's lymphoma and is related to PLZF, and ETO which is disrupted by the t(8;21)
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rearrangement in AML M2, have both been shown to bind N-CoR/SMRT (Dhordain et al., 1997; Wang et al., 1998a). This suggests that targeting of corepressor molecules to promoters by fusion genes, possibly compounded by sequestration of components of the corepressor complex which appear to be important for activity of Mad/Max which is linked to cellular differentiation (Laherty et al., 1997; He et al., 1998; Guidez et al., 1998), could provide an important final common pathway leading to oncogenesis.

A current working model summarising the potential mechanisms underlying development of APL associated with the t(15;17) and mediating the response to ATRA are presented in Figure 1.5. As discussed above, a wide range of experimental data has strongly implicated the PML-RARα fusion in the pathogenesis of APL; however, it is now clear that somewhat paradoxically the PML-RARα fusion protein is also critical for mediating the differentiation response to ATRA. This is supported by the finding that resistance in some APL NB4 cell lines reflects mutation within the ligand-binding domain of PML-RARα, associated with impaired hormone binding (Rosenauer et al., 1996; Shao et al., 1996). Interestingly, recent studies have demonstrated that similar mechanisms account for retinoid resistance in some patients with relapsed APL, previously exposed to ATRA (Imaizumi et al., 1998; Ding et al., 1998).

As to whether PML-RARα, PLZF-RARα, NPM-RARα and NuMA-RARα are alone sufficient to mediate APL or whether additional oncogenic events are required remains unclear. Expression of PML-RARα or PLZF-RARα in transgenic mice was found to lead to leukaemia in only a proportion of the animals, after a relatively long latency period (Brown et al., 1997; Grisolano et al., 1997, He et al., 1997; 1998). This prompted the suggestion that additional oncogenic events could be involved, fitting with mathematical models of leukaemogenesis (Vickers, 1996), with expression of the APL associated fusion proteins merely accounting for the phenotypic characteristics of the disease. Also it raised the possibility that the reciprocal fusion transcripts could play an important contributory role, particularly following the identification of an APL case with an RARα-PML fusion, apparently lacking a PML-RARα fusion gene (Lafage-Pochitaloff et al., 1995; Mozziconacci et al., 1998). As to whether additional oncogenic events are required remains to be determined; however it also remains a possibility that the long latency period observed in transgenic mouse models could indicate that the level of transgene expression and nature of the haemopoietic progenitor targeted do not fully reproduce events underlying the development of APL in man.
**Figure 1.5** Model for the pathogenesis of APL, and the response to retinoids
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1.7. CD2 expression in APL

1.7.1 Immunophenotype of APL: association of CD2 expression with the hypogranular variant subtype (M3v)

The immunophenotype of classical APL and M3v is broadly similar, being characterised by the presence of CD9, CD13, CD33 and sialylated CD15, with poor expression of HLA-DR (Avvisati et al., 1992; Paietta et al., 1994; Di Noto et al., 1994; Vahdat et al., 1994). In addition, a number of studies have highlighted the expression of the T cell antigen CD2 in a subgroup of patients with APL (Claxton et al., 1992; Biondi et al., 1995; Guglielmi et al., 1998). Interestingly, the initial report suggested a close relationship between the presence of a bcr 3 (5') PML breakpoint pattern and this phenomenon (Claxton et al., 1992). However, in this study and others it was clear that CD2 expression was not entirely restricted to patients with 5' breakpoints in PML (Claxton et al., 1992; Maslak et al., 1993). It subsequently transpired that the correlation with PML breakpoint was most likely accounted for by a close relationship between CD2 expression and the hypogranular variant form of APL, which is typified by a high presenting WBC (Avvisati et al., 1992) and has been associated with bcr 3 PML breakpoints in some studies (Biondi et al., 1992; Gallagher et al., 1997). The study performed by Biondi and colleagues also demonstrated that detection of CD2 in APL is a reflection of gene expression, rather than modulation of surface receptor (Biondi et al., 1995). A more recent, larger study by the same group involving 196 patients has established that CD2 expression occurs in 28% APL patients and confirmed the correlation with a bcr 3 PML breakpoint pattern and variant morphology (Guglielmi et al., 1998). In particular, 58% M3v cases were found to be CD2 positive, whilst only 18% with classical morphology expressed this antigen. Furthermore, a close relationship was found between CD2 positivity and expression of the stem cell marker CD34, and the B cell antigen CD19 (Guglielmi et al., 1998).

Therefore, whilst a number of studies have documented CD2 expression in APL, correlating it with a variety of disease characteristics, none has specifically addressed the molecular mechanisms underlying this phenomenon and its more general implications for the pathogenesis of this subtype of AML.
1.7.2 Implications of CD2 expression in APL

Mechanisms by which expression of lineage-specific and lineage-restricted surface antigens are co-ordinated during haemopoietic development are incompletely understood. Characterisation of these processes is clearly critical to an understanding of leukaemogenesis. Conversely however, considerable insight into the processes regulating haemopoiesis and underlying lineage commitment have been derived from characterisation of genes disrupted by leukaemogenic translocations and subsequent mouse models using transgenic, "knock-out" and "knock-in" approaches (reviewed by Rabitts, 1994; Tenen et al., 1997; Cross & Enver, 1997). These studies, together with the characterisation of factors interacting with regulatory elements of lineage restricted genes, suggest that networks of transcription factors play a key role in mediating lineage commitment. It would appear that haemopoietic growth factors play a largely permissive role in this process; although this remains an area of some contention (reviewed by Metcalf, 1998; Enver et al., 1998). Distinct experimental approaches have shown that a variety of genes whose expression is considered to be lineage restricted are co-expressed in multipotential haemopoietic progenitor cells (Ford et al., 1992; Jiménez et al., 1992; Cross et al., 1994; Ford et al., 1996). In particular, studies employing DNase I hypersensitivity assays established that the regulatory regions of β-globin, CD38, IgH and myeloperoxidase (MPO) genes lie within open chromatin in multipotential murine FDCP cells (Ford et al., 1992; Jiménez et al., 1992; Ford et al., 1996). These cells have the advantage that they can be induced to differentiate along various lineages with exposure to appropriate haemopoietic growth factors, thereby permitting investigation of the mechanisms underlying lineage commitment and consolidation of a given differentiation pathway. Interestingly, the IgH regulatory regions were found to become DNase I insensitive following induction of myeloid differentiation, whilst the CD38 enhancer remained sensitive under these circumstances, only becoming insensitive following progression along the B lineage pathway (Ford et al., 1992). Further investigation of the mechanisms underlying expression of MPO, has revealed that despite its accessibility to chromatin, the enhancer is functionally inactive in multipotential progenitors, becoming active only following commitment to the myeloid lineage (Ford et al., 1996). Pu.1 and C-EBP binding sites were identified in the enhancer, prompting the suggestion that regulation of MPO expression could be mediated through exchange of C-EBP isoforms at enhancer elements, which could reflect temporally regulated variations in expression level, cellular localisation and phosphorylation status, thereby influencing binding characteristics; and could also depend upon the phosphorylation status of Pu.1 (Ford et al., 1996). It may be envisaged...
that similar mechanisms may underly the regulation of other lineage restricted genes associated with commitment to a specific differentiation pathway. More recent studies have further extended the findings of DNase I hypersensitivity assays, which were performed in populations of multipotential murine haemopoietic progenitors, by employment of an RT-PCR approach to analyse expression patterns of arrays of lineage associated genes in single multipotential progenitor cells (Cheng et al., 1996; Hu et al., 1997). Whilst it remained a possibility that the results derived from DNase I hypersensitivity assays could have been explained by the presence of subpopulations of cells already committed to specific lineages, the single cell RT-PCR approach was able to confirm co-expression of β-globin, myeloperoxidase and various lineage associated cytokine receptors in multilineage progenitors, through analysis of both murine cell lines and CD34+/lin- selected marrow cells (Hu et al., 1997). Similarly, single-cell RT-PCR analysis of human CD34+/CD38- selected progenitors demonstrated co-expression of a number of lineage-restricted transcription factors, including Pu.1, GATA-2 and SCL (Cheng et al., 1996). These findings prompted a model whereby lineage commitment is determined by achievement of a threshold level of co-operating key transcription regulators which, re-enforced by positive and negative feedback loops, generate "programs" inducing and leading to maintenance of expression of the respective lineage associated genes, whilst mediating to varying degree repression of genes associated with other lineages (Cross & Enver, 1997; Enver & Greaves, 1998).

With more widespread application of immunophenotyping techniques to facilitate leukaemia diagnosis, it became apparent that antigens previously considered lymphoid-specific could be detected in cases of AML. For many years, it has been debated as to whether such "biphenotypic" leukaemias represent either expansions of rare stem cells that co-express lymphoid and myeloid antigens ("lineage promiscuity" model (Greaves et al., 1986)), or arise in lineage-committed cells in which the presence of "inappropriate" surface markers reflect aberrant gene expression associated with leukaemogenesis ("lineage infidelity" model (Smith et al., 1983)). In this context the presence of CD2 on some APL blasts is particularly intriguing since this subtype of AML is relatively well differentiated. Indeed, whilst studies performed in NOD/SCID mice have suggested that in the majority of cases of AML, the leukaemogenic event occurs at the level of primitive haemopoietic progenitors (CD34+/CD38-); blasts derived from APL patients failed to engraft in the mice suggesting that in this form of AML committed progenitors are targeted (Bonnet & Dick, 1997). Consistent with this hypothesis, CD34+/CD38-progenitors derived from patients with APL were found to be negative for the PML-
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RARα fusion gene, which was restricted to the committed CD34+/CD38+ marrow population (Turhan et al., 1995).

1.7.3 Regulation of CD2 expression

In order to begin to address the mechanism underlying CD2 expression in APL it is important to determine how the gene is regulated during normal haemopoietic development. The structure of the CD2 gene has been defined, comprising 5 exons spanning a genomic region of 15 kb (Diamond et al., 1988; Lang et al., 1988). DNase I hypersensitivity assays have established the presence of two distinct hypersensitive sites denoted DHS 1 & 2 situated 5' to the gene and a further series of sites lying 3' to the coding sequence, denoted DHS 3 (see Figure 1.6) (Wotton et al., 1989; Festenstein et al., 1996). DNase I hypersensitive sites are known to occur around regions of chromatin that are not tightly packed into nucleosomes, identifying the position of regulatory elements (Elgin, 1988; Gross & Garrard, 1988). A 28.5kb genomic fragment encompassing the CD2 coding sequence and DHS 1, 2 and 3 was found to permit position independent, copy number dependent, tissue specific (i.e. restricted to T cell lineage) expression in transgenic mice, thereby implying that all the key regulatory elements required for appropriate expression of the gene are contained within this region (Lang et al., 1988; Greaves et al., 1989). The 3' hypersensitive sites were found to correspond to enhancer elements and a locus control region (LCR), conferring high-level tissue specific expression of a CD2 transgene, independent of the site of integration (Greaves et al., 1989; Lake et al., 1990). A subsequent study using high resolution DNase I hypersensitivity assays has established that there are actually 3 clusters of hypersensitive sites within the 3' flanking region, denoted HS 1-3 (Festenstein et al., 1996). It is now clear that the LCR function is localised within the HS 3 region; which is essential to establish an open chromatin configuration and possesses no intrinsic enhancer activity. This would suggest that the LCR functions in the establishment &/or maintenance of an open chromatin domain acting in conjunction with adjacent tissue specific enhancers contained within HS1 of the DHS 3 region which are essential for mediating high level gene expression through an interaction with promoter elements (Festenstein et al., 1996; Kioussis & Festenstein, 1997).
**Figure 1.6**

A) Position of DNase I hypersensitive sites relative to the genomic structure of the CD2 gene as defined in T lymphoid cells (Wotton et al, 1989). 5' hypersensitive sites lie within the promoter (DHS 2) and upstream regulatory elements (DHS 1), whereas DHS 3 lies within the 3' enhancer region. "B" and "R" denote Bam HI and Eco RI restriction sites respectively. Black boxes denote exons.

B) Presence of DNase I hypersensitive sites in the CD2 gene as previously determined in haemopoietic and non-haemopoietic cell-lines (Wotton et al, 1989). DHS 3 was present in cells of T lineage irrespective of CD2 status, whilst DHS 1 & 2 were only detected in CD2 expressing cells. All sites were inaccessible to DNase I in B lymphoid and non-haemopoietic cells.
The 5' regulatory regions of the gene have been implicated in the control of the developmental stage at which CD2 is expressed, since the presence of DHS 1 and 2 was found to be closely correlated with CD2 expression in a variety of lymphoid cell lines (Wotton et al., 1989). DHS 2, which is situated immediately 5' of the transcriptional start site corresponds to the basal promoter (Outram et al., 1994). Minimal promoter activity required the presence of an E box motif, shown to bind USF, and a GATA box (possibly binding GATA-3) (Outram et al., 1994). Interestingly, Myc and Max were also found to be capable of binding the E box motif of the CD2 promoter, leading to transcriptional repression (Outram et al., 1994). DHS 1 is situated approximately 1.8 kb further upstream of DHS 2, corresponding to a region necessary for high level expression of CD2. Studies employing truncated promoter constructs derived from this region linked to a CAT reporter indicated the presence of an activator (or de-repressor) activity contained within the upstream region of DHS 1 and silencer element within the 3' region of this site. Further characterisation showed that integrity of an AP-2 binding site was critical to mediate the activator and silencer functions of this region (Outram, 1996).

Previous studies have revealed that 5' and 3' flanking regions of the CD2 locus are inaccessible to DNase I in CD2 negative Daudi and Bristol 8 B lymphoid cells and non-haemopoietic cells; whilst all hypersensitive sites are present in the CD2 positive Jurkat T cell line and IL-2 stimulated peripheral blood T lymphocytes (Wotton et al., 1989; Outram, 1996), see Figure 1.6. Interestingly, analysis of the CD2 negative KG1 cell line which has properties of a bipotential progenitor giving rise to sublines with T cell or macrophage characteristics (Ford et al., 1988), revealed that both upstream sites were closed, whilst DHS 3 was open (Wotton et al., 1989). However, the latter study did not address the chromatin configuration surrounding the CD2 gene in myeloid cell lines or in primary material derived from patients with acute or chronic leukaemia. A subsequent study established that the strength of the CD2 enhancer in various T lymphocytic cell lines was related to the developmental stage at which the cells had been arrested; strongest activity was noted in the mature T cell line Jurkat, whilst only low level activity was observed in KG1 cells and curiously also in the B cell line Daudi, whilst no activity was detected in non-haemopoietic cell lines (Lake et al., 1990).

Clearly further determination of the factors interacting with the regulatory elements of CD2 is an important first step to begin to address mechanisms by which expression might be detected in myeloid leukaemias such as APL. Determining these issues may provide insights into mechanisms determining commitment to and maintenance of a T
lineage phenotype and in addition could provide further information relating to the nature of haemopoietic progenitors targeted by the t(15;17) leading to APL.

1.8 Role of molecular techniques in the management of patients with APL

1.8.1 Molecular diagnosis of APL

It is now clear that APL requires a highly specific treatment approach in comparison to other forms of AML. This relates particularly to the significant risk of haemorrhagic death if the bleeding diathesis is not specifically addressed and the increased risk of relapse associated with poorer survival if these patients are not treated with ATRA in combination with chemotherapy (Tallman & Kwaan, 1992; Fenaux et al., 1994; Tallman et al., 1997). As discussed previously (section 1.6), the subgroup of APL patients likely to benefit from ATRA is essentially defined by the presence of the PML-RARα fusion gene. Therefore, establishing the presence of the PML/RARα rearrangement in suspected APL is critical not only for optimal patient management, but also meaningful analysis of clinical trials.

In the past, heavy reliance has been placed on cytogenetic analysis to confirm a morphological diagnosis of APL. In the majority of patients with this disease, the presence of an underlying PML/RARα rearrangement is indicated by the detection of the t(15;17). In early studies performed in specialised centres, it was claimed that the t(15;17) can be detected in all patients with APL (Larson et al., 1984). However, it is now clear that in some patients with underlying PML/RARα rearrangements the t(15;17) is not identified. In some instances this reflects a failure of cytogenetic technique; for example, direct chromosomal examination can lead to reporting of a falsely normal karyotype reflecting residual normal marrow elements whose growth is favoured relative to APL blasts (Berger et al., 1981). This shortcoming is usually overcome by more prolonged culture of leukaemic blasts prior to cytogenetic assessment, which has since been adopted as standard practice for analysis of cases with suspected APL. However, it is now clear that in some instances PML/RARα rearrangements reflect insertion events, whereby chromosomes 15 and 17 can be of normal appearance cytogenetically (Hiorns et al., 1994; Lafage-Pochitaloff et al., 1995). The frequency of cryptic PML/RARα rearrangements in APL has not previously been addressed, however the description of this phenomenon clearly implies that additional molecular techniques such as RT-PCR, PML-immunofluorescence or FISH are required to complement cytogenetics to confirm
a diagnosis of APL. RT-PCR affords a number of advantages. In particular it can be used to rapidly (cf. Southern analysis) determine PML breakpoint patterns (Borrow et al., 1992), which in some studies have been claimed to correlate with a variety of disease characteristics and provide independent prognostic information. The presence of a bcr 3 (5') PML breakpoint has been associated with an increased risk of relapse in some series (Borrow et al., 1992; Vahdat et al., 1994); whilst in other studies no such effect was seen (Fukutani et al., 1995; Gallagher et al., 1997). It is likely that these discrepancies are a reflection of relatively small sample sizes, compounded by inter- and intra- study variation in treatment approach. This issue clearly needs to be resolved, through analysis of larger, more uniformly treated groups of patients in the context of multicentre clinical trials, and could have a significant bearing on future treatment approaches should PML breakpoint be shown to be an independent prognostic variable. Also of interest was a recent report describing reduced ATRA sensitivity in a subgroup of patients with the rarer bcr 2 PML breakpoint (Gallagher et al., 1995). Molecular screening for PML-RARα and RARα-PML transcripts in large groups of patients entered into clinical trials, also affords the opportunity to address whether RARα-PML is likely to contribute to leukaemogenesis and influence the behaviour of the disease, as has recently been proposed (Lafage-Pochitaloff et al., 1995; Pandolfi, 1996). Furthermore, performance of RT-PCR in all patients with APL at diagnosis has the advantage of defining targets for subsequent minimal residual disease monitoring.

Since APL constitutes a haematological emergency, rapid diagnosis is essential. Whilst RT-PCR can be adapted to provide a result within 1 day (Diverio et al., 1996), there has been considerable interest in immunofluorescence techniques using polyclonal or monoclonal PML antisera as a rapid diagnostic test, confirming a diagnosis of APL through the characteristic microparticulate nuclear staining pattern (Dyck et al., 1995; Falini et al., 1997). However, PML-immunofluorescence has not been evaluated for the analysis of material derived from multicentre clinical trials and in addition provides an opportunity to address the role of PML nuclear bodies in APL cases with alternative translocations. Indeed, it is the very existence of these alternative translocations, whose characterisation has yielded significant advances in our understanding of APL pathogenesis; together with an appreciation of the prognostic value of cytogenetics in AML as a whole (Dastugue et al., 1995; Grimwade et al., 1998a), that suggest that adoption of molecular techniques should complement cytogenetic analysis, rather than precipitate its demise.
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1.8.2. Rationale for monitoring for minimal residual disease (MRD) in APL

Large scale clinical studies have established that the presence of the t(15;17), which is itself closely correlated with APL, defines a subgroup of AML patients with relatively favourable prognosis (Dastugue et al., 1995; Grimwade et al., 1998a). Attempts to improve cure rates in this group still further by intensifying consolidation therapy with the use of bone marrow transplantation in first complete remission (CR) proved unsuccessful, with benefit in terms of reduced relapse risk being offset by procedure related mortality (Burnett et al., 1998). However, the advent of retinoid therapy led to significant improvements in survival in APL (Fenaux et al., 1994; Tallman et al., 1997) and recent studies employing extended courses of ATRA in conjunction with combination chemotherapy have reported a marked reduction in relapse risk to levels of only 10-20% (Mandelli et al., 1997; Burnett et al., 1997). In order to achieve further improvements in outcome it has become increasingly important to identify the relatively small subgroup of patients at particular risk of relapse who may benefit from more intensive treatment in first remission.

Over the last few years there has been considerable interest in the possibility that monitoring for minimal residual disease (MRD), particularly using flow cytometric and PCR based techniques could potentially distinguish subgroups of patients with high and low risk of relapse. This has proved particularly fruitful in the context of CML, whereby rising numbers of BCR-ABL transcripts detected by nested RT-PCR following allogeneic BMT were found to be predictive of impending haematologic relapse, which could be prevented by clinical intervention using donor lymphocyte infusions (Van Rhee et al., 1994). In many instances molecular monitoring in patients with AML is more problematic, since at least 60% lack defined underlying chromosomal rearrangements suitable as targets for MRD detection (Grimwade et al., 1998a). This has led to considerable interest in flow cytometric techniques which are suitable for the evaluation of the majority of cases of AML. Such an approach can distinguish groups of patients at high and low risk of relapse by the presence of residual disease following consolidation therapy (San Miguel et al., 1997), however it is unclear whether this information is independent of other key prognostic indicators in AML such as cytogenetic risk group. Similarly, a number of studies using molecular or flow cytometric techniques demonstrated that persistence of residual disease in patients with ALL was associated with an increased risk of relapse (Brisco et al., 1994; Goulden et al., 1998; Coustan-Smith et al., 1998; Cuvé et al., 1998). The first of these studies also involved unselected groups of patients, again raising the possibility that MRD monitoring merely served to
identify patients at high risk of relapse who could have already been distinguished on the basis of pretreatment characteristics. This issue has been addressed by more recent studies performed in more uniform groups of ALL patients subdivided by pretreatment characteristics which have confirmed the predictive value of MRD monitoring and interestingly have indicated that the rate of disappearance of detectable disease following initiation of therapy is of independent prognostic significance. This would suggest that molecular monitoring could be used to determine treatment approach in this disease.

In contrast to previous studies involving patients with ALL; analysis of patients with APL, in common with CML, affords the opportunity to address the role of monitoring for MRD using a specific molecular marker in the context of a more homogeneous disease entity. Early studies suggested that this approach could predict outcome in APL and implied that PCR status following completion of therapy was of key prognostic significance (Lo Coco et al, 1992; Miller et al, 1992). Patients with persistent PCR detectable disease were found to have a poor prognosis, whilst patients in long term remission were found to lack detectable PML-RARα fusion transcripts using assays with sensitivity of 1 in 10^4 (Diverio et al, 1993). However, the original studies included significant numbers of patients treated with ATRA as single agent therapy or individuals treated in relapse, in whom in retrospect a high frequency of persistent PCR positivity associated with a poor long-term prognosis was not unexpected. It is now clear that combinations of ATRA and chemotherapy constitute the optimal therapeutic approach for APL at present and the majority of patients treated in this way ultimately become PCR negative using conventional PML-RARα assays (Miller et al, 1993; Fukutani et al., 1995). Therefore, the prognostic significance of molecular monitoring clearly needed to be reassessed in this context.

1.9. AIMS OF THIS THESIS

This thesis aimed to address a number of issues relating to the pathogenesis of APL and pertinent to the diagnosis and clinical treatment of patients with this condition. In particular it sought to evaluate the role of molecular analysis using RT-PCR and PML-immunofluorescence techniques, in comparison to more conventional methods such as karyotype assessment, as a means of clarifying diagnosis by identifying underlying PML/RARα rearrangements amongst a large group of patients entered into the MRC ATRA trial. It was hoped that this approach would reveal patients with cryptic
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*PML/RARα* rearrangements associated with APL and that characterisation of such cases would provide further insights into the relative roles of *PML-RARα* and *RARα-PML* fusion proteins in leukaemogenesis. RT-PCR permits rapid distinction of 5' and 3' breakpoint patterns in *PML*, therefore it was also of interest to determine whether breakpoint pattern is correlated with disease characteristics or outcome. Furthermore, it was also hoped that the MRC ATRA trial would permit characterisation of rare cases of APL with alternative translocations such as t(11;17)(q23;q21) associated with a *PLZF/RARα* rearrangement; thereby enabling one to determine whether disruption of PML nuclear bodies is critical to the pathogenesis of all forms of the disease. A further aim of this thesis was to investigate the role of molecular monitoring using nested RT-PCR for *PML-RARα* and *RARα-PML* fusion transcripts as a means of predicting prognosis in APL, affording the opportunity of evaluation of the prognostic significance of MRD monitoring in the context of a relatively homogeneous disease.

Over the last few years, a number of immunophenotyping studies have highlighted the expression of the T-lineage marker CD2 in a subgroup of patients with APL. The mechanisms underlying expression of lymphoid associated antigens in AML are currently unclear. Hence, the final aim of this thesis was to further characterise the regulation of CD2 in blasts derived from APL patients and haemopoietic cell lines by analysis of chromatin structure surrounding this gene using DNase I hypersensitivity assays.
CHAPTER 2: MATERIALS AND METHODS

A number of methods used have been described previously (Sambrook et al., 1989) and appropriate references are provided therein. Additional protocols were adapted from previously described methods as indicated in the relevant sections. For full details of the components of all buffers and solutions, please refer to section 2.17.

2.1. Preparation of DNA

2.1.1 Preparation of high quality cosmid DNA

_PML_ and _RARα_ cosmid DNA used as probes for FISH analysis of diagnostic material from patients with APL was derived from stabs from our laboratory (courtesy of K Howe). LB-agar plates supplemented with kanamycin (25μg/ml) were streaked with a sterile loop inoculated into the stab and incubated at 37°C for 16-18 hours. Single colonies were picked, inoculated into 50ml LB supplemented with 25μg/ml kanamycin and placed in a shaking incubator (New Brunswick Scientific, 150rpm) for a further 16-18 hours at 37°C, prior to DNA extraction. In order to prepare cosmid DNA of sufficient purity for direct sequence analysis or for use as probes for FISH analysis, QIAGEN purification columns were used, in accordance with the manufacturer's instructions (QIAGEN Inc., USA). _PML_ and _RARα_ cosmid DNA used for FISH analyses was initially characterised by restriction enzyme digestion (_EcoRI_). Specificity was subsequently confirmed by hybridisation of _PML_ and _RARα_ cosmid probes to 15q and 17q, respectively on metaphase spreads with a normal karyotype and the demonstration of a fusion signal localised to 15q in cases of APL associated with the t(15;17). All FISH experiments were performed by P Gorman, ICRF using previously described methods (Borrow et al., 1994).

2.1.2 Preparation of genomic DNA

Details of the method used to extract genomic DNA from DNase 1 treated and untreated haemopoietic cell nuclei as part of the DNase I hypersensitivity assay is provided in section 2.6.
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2.1.3 Preparation of oligonucleotides

Oligonucleotides were obtained from the Imperial Cancer Research Fund Oligonucleotide Synthesis Service (ICRF, Clare Hall Laboratories, South Mimms), supplied in 0.04μM quantities fully deprotected and dried down. Side-products were removed by ethanol precipitation. In order to avoid risk of contamination of primers with PCR products, oligonucleotides were always prepared in a laminar-flow hood in a different room from that in which PCR reactions were set up. Furthermore, all solutions were made up with sterile water. Oligonucleotides were dissolved in 200μl 0.3M NaOAc, 10mM MgCl2, and precipitated by the addition of 600μl cold ethanol. After 15 minutes at -70°C, tubes were centrifuged at 13,000rpm (11,600g) for 20 minutes (Heraeus Biofuge) at room temperature. The oligonucleotide pellet was washed in 80% ethanol; after air drying the pellet was resuspended in TE at a concentration of 1μg/μl (stock solution). Aliquots of stock solution were diluted in sterile water at a concentration of 65ng/μl for use in standard PCR reactions (working solution) and at a concentration of 10ng/μl for use in cycle sequencing reactions for automated sequencing (see sections 2.7, 2.8.2 and 2.11.2, respectively). All oligonucleotide solutions were stored at -20°C.

2.2. Preparation of RNA

2.2.1 Preparation of peripheral blood/bone marrow samples and haemopoietic cell lines

Bone marrow/peripheral blood samples were dispensed into 15ml sterile tubes and centrifuged at 1600g for 5 minutes. After removal of the plasma/supernatant with a sterile pipette, the buffy coat layer was gently removed and transferred to a new 15ml tube. Isolation of mononuclear cells with this technique was used in preference to employment of a Ficoll gradient (Ficoll-Paque, Pharmacia) due to greater efficiency for samples with relatively low cell counts. At this stage a drop of the buffy coat was used to prepare smears on 3 microscope slides, 1 was May-Grünwald-Giemsa (MGG) stained for morphological assessment and 2 were stored in aluminium foil at -20°C for subsequent analysis by immunofluorescence (see section 2.12). The buffy coat was then resuspended to a volume of 10ml in Dulbecco’s PBS, and a small aliquot taken to determine the number of cells available for RNA extraction (STKS, Coulter). The resuspended buffy coat was centrifuged at 1600g for 5 minutes at room temperature, after removal of the supernatant, the cell pellet was resuspended in GTC-ME causing 62
denaturation and dissociation of nuclear proteins from nucleic acids due to loss of secondary structure and inactivation of RNAases. The volume of GTC-ME was dependent on the number of PBS suspended cells: for 1-2 × 10^7 GTC-ME was added to a final volume of 1ml, for >2-4 × 10^7 GTC-ME was added to 2ml and for >4-6 × 10^7 cells to a volume of 3ml, etc. Cells in GTC-ME were stored at 4°C until extraction of total RNA.

Total RNA was also prepared from haemopoietic cell lines, used as controls for RT-PCR assays performed on material derived from patients with suspected APL. Cell lines used for this purpose included NB4 (gift from M. Lanotte, Hôpital St Louis, Paris) which carries the t(15;17) leading to the formation of PML-RARα and RARα-PML fusion transcripts (Lanotte et al., 1991; Borrow et al., 1992) and the AML M2 cell line HL60 (Dalton et al., 1988), which lacks a PML/RARα rearrangement (Borrow et al., 1992). Total RNA was extracted from 1 × 10^7 cultured cells, details of the tissue culture conditions are provided in section 2.14. Cells were harvested at 1,100 rpm for 5 minutes and cell pellet washed in 50mls PBSA. Cells were washed one further time in PBSA before the cell pellet was resuspended in GTC-ME to a final volume of 1ml.

2.2.2 Preparation of RNA from haemopoietic cell lines and clinical material

Total RNA was prepared using a method based on that previously described by Chomczynski & Sacchi (1987). To 1ml cells in GTC-ME (i.e. 1-2 × 10^7 cells), 100μl 2M NaOAc, 1ml phenol and 200μl chloroform/isoamyl alcohol were added to achieve DNA precipitation and disruption of nucleoprotein complexes. Samples were mixed by vortexing, cooled on ice for 15 minutes and centrifuged at 11,600g for 20 minutes at 4°C. The aqueous phase was retained, to which was added 600μl isopropanol to precipitate the RNA; samples were then left at -20°C for at least 1 hour. Samples were centrifuged at 11,600g for 20 minutes at 4°C, the supernatant was discarded and RNA pellets washed in 500μl 75% ethanol. 500μl GTC-ME was added to redissolve, followed by 600μl isopropanol to reprecipitate the RNA; samples were again left at -20°C for at least 1 hour. Samples were centrifuged and RNA pellets washed in 75% ethanol as before. RNA was air dried briefly, then dissolved in 50μl DEPC-treated sterile water and stored at -70°C.
2.2.3 Assessment of total RNA quality

Prior to storage, RNA concentration and quality were determined by optical density (OD) measurements (see section 2.3.) and gel electrophoresis (3μl RNA in DEPC water run in 0.5% w/v agarose). Gel electrophoresis served to validate RNA concentration by OD measurement, determined the degree of DNA contamination and assessed RNA integrity and quality by the presence of 18S and 28S ribosomal bands.

2.3. Quantification of DNA and RNA

2.3.1 Quantification by spectrophotometry

Quantification of DNA or RNA was determined by spectrophotometric measurement of the absorbence of UV light by nucleic acid diluted in sterile water at a wavelength of 260nm. A ratio of readings was taken at 260 and 280nm, providing an indication of purity (260/280 ratio values falling within the range 1.80 - 2.00 were considered satisfactory). Nucleic acid concentrations were calculated on the basis that an OD$_{260}$ of 1.0 corresponds to 50μg/ml double stranded DNA, or 40μg/ml single stranded RNA.

2.3.2 Quantification by gel analysis

DNA concentrations were also estimated by comparison of intensity of DNA fragments relative to known concentrations of DNA size marker e.g. Hind III digested λ DNA (Pharmacia) loaded onto the gel.

2.4. Size separation of DNA by agarose gel electrophoresis

DNA was size separated by electrophoresis through agarose gels in Bio-rad DNA sub cell tanks (Bio-rad, Hemel Hempstead, UK) containing 1x TAE as buffer. Agarose gel concentration was selected according to the average DNA fragment size; for separation of PCR products/DNA fragments of <1000bp gels comprising 1.5% agarose (Gibco, Life Technologies) in 1x TAE buffer were used, whilst for larger fragments and DNase I hypersensitivity assays 1% gels were employed. DNA was loaded into wells with 5-7μl 5x Ficoll-blue loading buffer. Size of DNA fragments was estimated in relation to the
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migration of the following markers: 100bp ladder (Pharmacia) for PCR products and Hind III digested λ DNA (Pharmacia), Eco RI/Hind III digested λ DNA (NBL Gene Sciences Ltd) and 1kb ladder (Pharmacia) for restriction enzyme digests of plasmid/cosmid DNA and DNase I hypersensitivity assays. DNA was visualised by incorporation of ethidium bromide (0.1μg/ml) into the gel and photographed under UV light with a DS 34 Polaroid camera and Polaroid 667 film or Kodak Digital Science DC40 camera with image viewing by means of the BioMax ID Image Analysis software (Kodak).

2.5. Restriction enzyme digestion of DNA

DNA was digested at 37°C with restriction enzymes (New England Biolabs, Northumbria Biologicals Ltd), in the presence of the appropriate buffer in accordance with the manufacturer's instructions. Digests were typically performed in a 20μl or 50μl reaction. The number of units of enzyme added and length of digestion were calculated on the basis that 1 unit digests 1μg DNA/hour, with a margin of error to ensure complete digestion. Plasmid/cosmid DNA was digested for 1-2 hours, whereas for genomic DNA used for DNase I hypersensitivity assays a more prolonged incubation period of 8-9 hours was employed (see section 2.6.).

2.6. DNase I hypersensitivity assays

DNase I hypersensitivity assays were performed, based on the method of Wotton et al., (1989) using a variety of haemopoietic cell lines, peripheral blood T lymphocytes (cultured in the presence of 2μg/ml phytohaemagglutinin (PHA), 20ng/ml IL-2, courtesy of V.Morales, D.Cantrell, ICRF) and cells derived from patients with newly diagnosed (untreated) acute or chronic leukaemia (see section 2.16). Typically 2-7 x 10⁸ cells were used for each assay; cultured cell-lines or T lymphocytes were initially pellet ed at 1100rpm and then washed with PBSA. For analysis of bone marrow or peripheral blood samples, leucocytes were separated by centrifugation, employing a Ficoll density gradient (Ficoll-Paque, Pharmacia). Cells were washed in PBSA, counted, assessed for viability using trypan blue exclusion and aliquots of 1 x 10⁷ cells frozen in 1ml freeze mix (see sections 2.13, 2.14) for subsequent immunophenotyping. The remainder of the patient derived cells/cultured haemopoietic cells (2-7 x 10⁸) were gently agitated in 2ml hypotonic buffer (IB) on ice for 30 minutes, then lysed with 1ml 5% Nonidet P-40
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constituted in IB, and subsequently disrupted by homogenisation with a loose pestle. Cell lysis was confirmed by phase contrast microscopy. Nuclei were centrifuged through an 8.5% (w/v) sucrose cushion at 750rpm and resuspended in 1ml of RSB buffer per digestion. Nuclei were then digested with various amounts of DNase I (Sigma, range 0.0-8.0 µg, dependent on initial cell count). After incubation for 5 minutes at 37°C, the enzyme reaction was terminated and nuclei lysed by addition of 1ml "stop" buffer and tubes returned to ice. 35µl of each sample was separated by electrophoresis through a 1% agarose gel to determine the success of DNase digestion. The remainder of the samples was treated with 40µl (200µg/ml) proteinase K (Sigma) and incubated at 37°C for 16-18 hours. The sample derived from the untreated nuclei and samples encompassing a suitable range of DNase digestion were selected for DNA extraction. This was undertaken using a 50:50 mixture of phenol and chloroform until the aqueous layer was clear, with subsequent chloroform extraction. DNA was precipitated using 3M NaOAc (1/10th volume) and absolute ethanol (2.5x volume) and subsequently washed in 70% ethanol. DNA was resuspended in TE and concentration determined by UV spectrophotometry (section 2.3.1). 10µg-15µg of DNA derived from each of the DNase digestions and the untreated nuclei was digested with Bam HI for demonstration of hypersensitive sites in the 5' flanking region of CD2, and Eco RI for analysis of 3' sites (see Figure 1.6). Enzyme digests were performed for 8-9 hours at 37°C in a 50µl reaction; 40u restriction enzyme was used initially, with a further 40u added after 4 hours to ensure complete digestion. Restriction digests were separated by electrophoresis through 1% agarose gels and transferred to Hybond N+ filters (Amersham International). Filters were probed using [α-32P]dCTP (Amersham International) random labelled 380bp Eco RI,Ssp I CD2 cDNA fragment (probe A) to detect 5' hypersensitive sites and an 880bp Eco RI, Nde I CD2 cDNA fragment (probe B) to detect 3' hypersensitive sites (Wotton et al., 1989) (see Figure 5.3). CD2 cDNA in pUC13 was obtained courtesy of S.V.Outram, ICRF. Filters were subsequently rehybridised using probes (L1 and ωα, see section 5.2) from the region 5' to the α-globin gene; these probes serve to detect constitutive hypersensitive sites (Craddock et al., 1995), thereby providing a control for the success of DNase I and restriction enzyme digestion in each experiment.

2.7. Amplification of genomic DNA using the polymerase chain reaction (PCR)

Cosmid/PAC derived DNA was amplified by PCR, using a Techne Programmable Dri-block (PHC-1) or Hybaid Omnigene Thermocycler. 50-100ng DNA template was added
to a reaction mixture comprising 5\(\mu\)l 10x \textit{Taq} buffer with 15mM MgCl\(_2\) (Promega), each dNTP (Pharmacia Biotech) at 100\(\mu\)M, 20pmol of each primer, 1\(\mu\) l \textit{Taq} polymerase (Promega), 1.25\(\mu\)l DMSO (BDH Laboratory Supplies) and sterile distilled water to make the total volume up to 50\(\mu\)l. PCR reactions were then overlaid with 30\(\mu\)l liquid paraffin. Cycling conditions were generally 1 minute denaturation at 95\(^\circ\)C, 1 minute annealing at 55\(^\circ\)C and 1 minute extension at 72\(^\circ\)C, repeated for 35 cycles, followed by a final extension period of 72\(^\circ\)C for 10 minutes. Extension times were altered according to the predicted size of PCR product. Extreme care was taken to avoid contamination of component solutions with PCR products; PCR mixtures were invariably set up in a laminar flow hood in a separate laboratory from that in which DNA template was added. Gloves were changed frequently and sterile plugged pipette tips used throughout. Water (no template) controls were routinely included.

2.8. Nested reverse transcriptase polymerase chain reaction

2.8.1 Preparation of cDNA

Nested RT-PCR was performed using total RNA prepared from cell lines or bone marrow/peripheral blood samples taken from patients with suspected APL (see section 2.16). RT-PCR assays performed on patient derived material were routinely accompanied by a series of controls designed to confirm the efficacy of the reverse transcription (RT) step, ensure adequate sensitivity and exclude contamination at all stages of the RT-PCR procedure. These included RNA derived from a 1 in 1000 dilution of the NB4 cell line in \textit{PML-RAR}\(\alpha\) negative filler cells (HL60) which served both as a positive and sensitivity control in the subsequent PCR reaction, RNA from a non-APL patient/cell line e.g. HL60 and a control lacking RNA template (RT water control). cDNA was prepared from 1\(\mu\)g total RNA, using 0.5\(\mu\)g 18-mer oligo-dT (oligonucleotide synthesis service, ICRF) as primer in a 20\(\mu\)l reaction. RNA, oligo-dT and sterile water were heated at 65\(^\circ\)C for 5 minutes, samples were spun briefly at 11,600g and placed on ice prior to the addition of the reaction mixture. This comprised 10\(\mu\)l AMV reverse transcriptase (Northumbria Biologicals Ltd) in the manufacturer's buffer, 10\(\mu\)l RNasin (Promega) and 1mmol of each dNTP (Pharmacia). Samples were incubated for 1 hour at 42\(^\circ\)C, at which stage the reaction was stopped by the addition of 100-200\(\mu\)l TE, tubes were then transferred to ice as the PCR stage was set up. Residual cDNA was stored at -20\(^\circ\)C.
Figure 2.1

Flow diagram demonstrating protocol for nested RT-PCR to detect PML/RARα rearrangements in APL, and subsequent distinction of PML breakpoint patterns. Primers used are shown in Figure 2.2.
Schematic representation of primer positions employed for nested RT-PCR relative to genomic structure of PML and RARα, and breakpoints generated by the t(15;17). Positions of the most frequent breakpoints within PML are indicated by vertical arrows. Disruption of RARα invariably occurs within intron 2. Alternative splicing between PML central exons leads to amplification of multiple PML, PML-RARα (bcr 1, bcr 2) and RARα-PML (bcr 3) fusion products, as shown in Figures 3.1 & 3.5.

**PRIMERS:**

**PML**
1. 5'-AGCTGCTGGAGGCTGTGGAC-3'
2. 5'-TGTGCTGCAGCGCATCCGCA-3'
3. 5'-CTGCTGATCACCACAACGCG-3'
4. 5'-CGGCATCTGAGTCTTCCGAG-3'
9. 5'-AGTGTACGCCTTCTCCATCA-3'

**RARα**
5. 5'-GGCCAGCAACAGCAGCTCCT-3'
6. 5'-GGTGCCTCCCTACGCCTTCT-3'
7. 5'-GGCGCTGACCCCATAGTGGT-3'
8. 5'-TCTTCTGGATGCTGCGGCGG-3'

**RARα1**
5. 5'-GGCCAGCAACAGCAGCTCCT-3'
6. 5'-GGTGCCTCCCTACGCCTTCT-3'
7. 5'-GGCGCTGACCCCATAGTGGT-3'
8. 5'-TCTTCTGGATGCTGCGGCGG-3'
2.8.2 Nested PCR from cDNA template

Four separate nested RT-PCR reactions were routinely performed in parallel for samples derived from patients with suspected APL, for cell-line controls as well as water controls for the RT and PCR steps. Primer sets were used to detect normal \textit{PML} and \textit{RAR\alpha} transcripts as controls for the success of reverse transcription and RNA integrity, in addition to combinations of primers to detect \textit{PML-RAR\alpha} and \textit{RAR\alpha-PML} fusion transcripts as indicated in Figures 2.1, 2.2. All PCR experiments were performed at least twice from the RT stage and results only considered reliable provided \textit{PML} and \textit{RAR\alpha} transcripts were detected and all other controls were satisfactory. The first round reaction mixture comprised 10\mu l cDNA, 5\mu l 10x PCR buffer with 15 mM MgCl\textsubscript{2} (Promega), each dNTP at 100\mu M, 20pmol of each external primer, 1u of \textit{Taq} polymerase and sterile water to make the total volume up to 50\mu l. Sufficient reaction mixture for each of the 4 separate amplifications for patient samples and controls was prepared and added to sterile PCR tubes prior to addition of the cDNA and subsequent overlay of 30\mu l liquid paraffin. Tubes were transferred to the PCR machine, cycle conditions were as described in section 2.7. The second round PCR reaction mixture included 1\mu l first round product and 20pmol of each internal primer; concentrations of PCR buffer, dNTPs and \textit{Taq} polymerase were identical to the first round PCR, and again sterile distilled water was added to bring the total volume to 50\mu l. Reaction mixtures were overlaid with paraffin oil and samples transferred to the PCR machine, identical cycling conditions were used as described for the first round. After gel electrophoresis of 20\mu l second round product (see section 2.4), PCR products were routinely transferred to filters for hybridisation with a 221bp \textit{RAR\alpha} probe (H7b), an \textit{Xho I}, \textit{Kpn I} cDNA fragment that traverses the \textit{RAR\alpha} breakpoint site, as previously described (Borrow et al., 1990). This probe hybridises to both \textit{PML-RAR\alpha} and \textit{RAR\alpha-PML} fusion transcripts, serving to confirm specificity of PCR products and also to confirm results determined to be negative by gel electrophoresis. In selected cases probes hybridising to 5' (U3,400) and 3' (U3,1.3) regions of \textit{PML}, (courtesy of Dr A.Goddard, Goddard et al., 1991) were also used. For APL cases found to have 3' breakpoints in \textit{PML}, the second round of PCR was repeated using an internal primer (primer 9) derived from \textit{PML} exon 5 (see Figure 2.2) which facilitates distinction of bcr 1 from bcr 2 breakpoints, by subsequent oligonucleotide probe hybridisation and/or sequence analysis of PCR products (see Figure 2.1, section 2.9.2, 2.11).

In cases of suspected APL lacking \textit{PML/RAR\alpha} rearrangements, nested RT-PCR was also performed to detect \textit{PLZF-RAR\alpha} and \textit{RAR\alpha1-PLZF} fusion transcripts. Methods were as
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described above, except for the substitution of PLZF for PML primers and use of a higher annealing temperature of 57°C. PLZF primers used were designed from the published sequence, taking into account breakpoint positions identified in previously described cases of t(11;17)(q23;q21) associated APL (Chen et al., 1993a,b; Licht et al., 1995). For amplification of PLZF-RARα, PLZF primers were as follows:

external - 5'-TCCAGAGGGAGCTGTTCAGC-3'
internal - 5'-TCGAGCTTCCTGATAACGAG-3'

For amplification of RARα1-PLZF, the following PLZF primers were used:

external - 5'-ATGTCAGTGCCAGTATGGGT-3'
internal - 5'-CACTGATCACAGACAAAGGC-3'

Rigorous steps were taken to avoid contamination at all stages of the RT-PCR procedure:

1) Patient samples were processed and RNA synthesised in a different building (University College Hospital, London) from that in which RT-PCR was performed (ICRF, Lincoln's Inn Fields or Guy's Hospital, London).

2) PCR reaction mixtures were aliquoted in a laminar flow hood in a separate laboratory from that in which template was added and gel electrophoresis was performed. No PCR products or clones of PML, RARα, PML-RARα or RARα-PML were permitted within or near the laminar flow hoods. Indeed no clones were handled in the laboratory at times in which RT-PCR was performed.

3) PCR products were briefly centrifuged at 11,600g prior to manipulation to reduce contamination due to aerosols and gloves were changed after handling of tubes from different patients.

4) Sterile plugged tips were used throughout.

2.9 DNA analysis by Southern hybridisation

2.9.1 Southern transfer of DNA

After size separation by electrophoresis in agarose gels, the DNA was transferred to nylon filters (Hybond N+, Amersham) by alkali transfer as previously described (Sambrook et al., 1989). Gels of genomic DNA digests were initially nicked with 0.25M HCL for 10-15 minutes to improve transfer of large fragments, washed with distilled water and subsequently neutralised with 0.4M NaOH. Transfer was performed overnight and filters subsequently washed three times in 2x SSC, prior to storage at 4°C wrapped in Saran wrap.
2.9.2 Radioactive labelling of DNA probes

DNA probes for hybridisation were labelled by one of two techniques. Generally, oligo-labelling was used; however for short oligonucleotide probes, end-labelling was the chosen technique.

*Generation of probes*

DNA used as probes included fragments derived from restriction enzyme digestion of plasmid DNA. Digested DNA was electrophoresed through 0.8-1% low melting point (LMP) agarose; appropriate fragments were excised under UV visualisation, resuspended in an equal volume of TE and stored at 4°C. Single-band PCR products generated from genomic or cDNA were also used as probes. PCR products were diluted in sterile water (dependent upon band intensity) and 30μl purified by spinning down Microspin™ S-400 HR columns (Pharmacia Biotech) to remove excess primers and dNTPs in accordance with the manufacturer's instructions. Oligonucleotide probes were prepared as described in section 2.1.3.

*Oligo-labelling of DNA probes*

DNA probes generated to check the specificity of *PML-RARα* and *RARα-PML* PCR amplification products and for use in DNase 1 hypersensitivity studies of the *CD2* gene were labelled with [α-32P]dCTP (Amersham International) by the random priming method (Feinberg & Vogelstein, 1984). 30-50ng of DNA was boiled for 5 minutes in the correct volume of distilled water for a total reaction volume of 50μl. When DNA fragments in LMP agarose were used, this step was preceded by heating of probe DNA at 65°C for 10 minutes to melt the agarose; the DNA volume did not exceed 25% of the total volume and if necessary a 100μl reaction volume was selected. After brief spinning and placement on ice, for a 50μl reaction: 10μl 5x oligo-labelling buffer (OLB), 1.0μl BSA (10mg/ml) and 2.5μl [α-32P]dCTP (25μCi) were added, followed by 1.0μl DNA polymerase Klenow fragment (1U/μl) and mixed gently, prior to incubation for at least 2 hours at 37°C or overnight (15 hours) at room temperature. The reaction was stopped by addition of 50μl TE 8; unincorporated nucleotide was subsequently removed by spinning through a Sephadex G50 column. Labelling was considered satisfactory with at least 500 counts/second/μl. Probes lacking repetitive sequences were subsequently denatured with 1M NaOH for 5 minutes at room temperature (volume added = 25% volume of probe), prior to addition to the hybridisation solution. For probes containing repetitive sequences, labelled DNA was boiled in the presence of 20μl (198μg) human placental
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DNA (Sigma) for 5 minutes and then incubated at 65°C for 1-2 hours before adding to the hybridisation solution.

End-labelling

PCR products derived from cases of APL with PML/RARα rearrangements associated with disruption within the 3’ region of PML were hybridised with an oligonucleotide probe spanning the RARα exon 3-PML exon 6 junction (5’-TCTCAATGGCTGCCTC-3’) in order to distinguish bcr 1 and bcr 2 breakpoints, using a method based on that described by Gallagher et al. (1995). For a 50μl reaction, 1μg oligonucleotide was end-labelled with 30μCi [γ-32P] dATP (Amersham International) in the presence of 5μl 10x polynucleotide kinase (PNK) buffer (Promega) and 2μl T4 PNK (Promega). The reaction mixture was incubated at 37°C for 2 hours; unincorporated nucleotide was subsequently removed by spinning down a sephadex G25 column. Labelling was considered satisfactory with at least 500 counts/second/μl. No denaturation step was required and probes were subsequently added directly to the hybridisation solution.

2.9.3 Prehybridisation and hybridisation of DNA probes to filters

Filters were pretreated with hybridisation solution in bottles (Hybaid, UK) for at least 2 hours at 65°C. After discarding the prehybridisation solution, filters were hybridised with labelled probe added to 20-25mls hybridisation solution overnight at 65°C. Filters were washed for 15 minute intervals at 65°C, the first wash comprised 1x SSC, 0.1% SDS; filters from DNase I hypersensitivity assays were subsequently washed to final stringency of 0.5x-0.2x SSC, 0.1% SDS according to the degree of radioactive signal, whilst filters derived from RT-PCR experiments were washed to a final stringency of 0.1x SSC, 0.1% SDS. Filters from RT-PCR experiments were exposed to Kodak XAR or Fuji RX film for 1-12 hours, between intensifying screens at -70°C. For DNase I hypersensitivity experiments Kodak XAR film was used with exposure times of 3-14 days.

2.9.4 Prehybridisation and hybridisation of oligonucleotide probes to filters

Procedures were as described above (section 2.9.3), however prehybridisation and hybridisation were performed at 42°C. Filters were then washed in 6x SSC, 0.1% SDS for 30 minute intervals initially at 42°C, to a final temperature of 51°C prior to exposure to Kodak XAR film between intensifying screens for 1.5-3 hours at -70°C.
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2.10. Northern analysis

2.10.1 Electrophoretic size separation of RNA

Samples of total RNA (2μg) were diluted in 10μl RNA loading buffer, heated at 65°C for 5 minutes, then cooled on ice prior to addition of 4μl RNA loading dye. RNA was then separated electrophoretically through 1% agarose gels incorporating 1% formaldehyde in 1x RNA gel running buffer. RNA was visualised by staining the gel with ethidium bromide (0.5μg/ml). RNA was subsequently transferred to Hybond N (Amersham International) using 20x SSC; filters were then washed in 2x SSC and stored at 4°C in Saran wrap.

2.10.2 Prehybridisation and hybridisation of Northern filters

Filters were prehybridised using 20mls prehybridisation solution (for Northern analysis, see section 2.17) for 24 hours at 42°C, this was then replaced with fresh hybridisation solution containing the radiolabelled DNA probe. These were labelled as described in section 2.9.2; probes were denatured by boiling for 5 minutes prior to addition to the hybridisation solution which had been preheated to 42°C. Filters were then hybridised at 42°C for 18-19 hours.

2.10.3 Washing of Northern filters

Filters were washed twice (10 minutes each wash) in 2x SSC, 0.1% SDS at room temperature, followed by a final 20 minute wash in 0.1x SSC, 0.1% SDS at 50°C. Filters were then shaken to remove excess wash solution, wrapped in Saran wrap and exposed to Kodak XAR film between intensifying screens at -70°C for at least 1 week.

2.11. Sequence analysis of DNA

All sequence analyses were performed using an ABI 373 or 377 automated DNA sequencer (Applied Biosystems).
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2.11.1 DNA preparation

PCR products for sequence analysis were initially diluted (generally 10μl in 20μl sterile water, dependent on band intensity), then purified using Microspin™ S-400 HR columns (Pharmacia Biotech) in accordance with the manufacturer's instructions. Sequence analysis of plasmid or cosmid DNA was undertaken using material prepared using QIAGEN columns (see section 2.1.1).

2.11.2 Preparation of sequence reactions

Cycle sequencing reactions were performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK) in accordance with the manufacturer's instructions. In general, 200ng purified PCR product, or 2μg cosmid DNA was sufficient for a 20μl reaction. DNA was added to a reaction mixture comprising 3.2 pmoles primer and premix solution (Applied Biosystems) composed of dNTPs, fluorescently labelled dye deoxy terminators and Taq polymerase in buffer. Conditions for cycle sequencing were as follows: 96°C for 30 seconds, 50°C for 30 seconds and 60°C for 4 minutes repeated for 25 cycles in a PTC-100 PCR machine (MJ Research Inc.). Excess dye-labelled terminators were removed from the products by ethanol precipitation in accordance with the manufacturer's instructions (Applied Biosystems). After washing in 70% ethanol, pellets were air dried, then resuspended in loading buffer (5 parts deionised formamide : 1 part 50mM EDTA, pH 8.0, supplemented with dextran blue to facilitate sample visualisation).

2.11.3 Preparation and running of sequencing gels

Samples were heat denatured, then separated on a 4.75% polyacrylamide gel for 14 hours at 2,500 Volts, 40 mAmps, 30 Watts using an ABI 373A automated DNA sequencer, in 1x TBE buffer in accordance with the manufacturer's instructions. More recently, after heat denaturation samples were separated on a 4.25% polyacrylamide gel for 7 hours using an ABI 377 automated sequencer using the 2X sequencing program. Data was collected and analysed using the Data Collection and Sequencing Analysis programs (Applied Biosystems).
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2.11.4 Computer analysis of DNA sequences

Sequences were initially edited using the Sequencing Analysis or Editview programs (Applied Biosystems). Additional analyses were undertaken using the Wisconsin Package version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin. Alignments were performed using BESTFIT and GAP programs, whilst FASTA and TFASTA programs were used for homology searches.

2.12. Immunofluorescence

2.12.1 Immunofluorescence using PML polyclonal antisera

Patient material used for PML-immunofluorescence studies included smears of peripheral blood, bone marrow or buffy coat preparations made from samples received for molecular analyses, stored unfixed on microscope slides at -20°C for up to 2 years. For analysis of cell lines as controls and material derived from patients used to determine the in vitro differentiation response to ATRA, cytospin preparations were used. All samples were fixed in methanol at -20°C for 15 minutes, washed in PBSA and incubated at room temperature for 1 hour with PML polyclonal antiserum (raised against 15-1745bp PML (Borden et al., 1995; Boddy et al., 1996), kindly provided by N. Boddy, K. Howe, ICRF, London) at 1/500 dilution in PBSA containing 10% newborn calf serum (Gibco) and 0.5% NP40 (Sigma). Slides were then washed three times with PBSA for 5 minutes each, prior to incubation for 30 minutes with anti-rabbit FITC conjugated secondary antibody at 1/200 dilution (Dako Ltd). Slides were washed as before, then mounted in Citifluor (Agar, product no. R1320). Slides were viewed by fluorescence microscopy at 495 nm (excitation λ) and 525 nm (emission λ) in conjunction with phase contrast using a Biorad M.R.C. 600 or 1000 confocal microscope and the Comos image collection program (Biorad). Image manipulations were performed using the Photoshop software (Adobe). All patient and cell line slides were coded and reported "blind" by 2 individuals with concordant results (D.Grimwade with Dr Lynne Davies or Dr Estelle Duprez). In non-APL cells the PML antiserum typically detects less than 30 discrete nuclear speckles, whilst in APL cells with the t(15;17) the antibody which recognises both PML and PML-RARα detects a characteristic microparticulate nuclear staining pattern (Daniel et al., 1993; Dyck et al., 1994; Weis et al., 1994; Boddy et al., 1996). At least 100 cells were counted for each
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sample; samples in which at least 15% cells demonstrated >30 nuclear dots were reported to have a microparticulate staining pattern.

2.12.2 Dual immunofluorescence

Slides derived from cases of APL in which the classic t(15;17) was not identified by cytogenetics, as well as a series of controls with other forms of AML were subjected to dual immunofluorescence studies using PIC1 polyclonal antibody (courtesy of N.Boddy, K. Howe, ICRF, described Boddy et al., 1996) and PML monoclonal antibody (PG-M3 which detects PML and PML-RARα, described by Flenghi et al., 1995, courtesy of P.G. Pelicci, European Institute of Oncology, Milan). Slides were fixed in methanol at -20°C for 15 minutes as before, then allowed to air dry and preblocked with 10% fetal calf serum. Slides were then incubated simultaneously with PG-M3 and PIC1 antibodies at a dilution of 1/5 and 1/200 respectively, in 0.5% Tween in PBSA for 1 hour at room temperature. All incubations were followed by 3 washes in PBSA, followed by a final wash comprising 0.05% Tween in PBSA for 10 minutes. Preparations were examined by confocal laser scanning using MRC inverted confocal microscope as described above.

2.13. Immunophenotyping

2.13.1 Immunostaining

Immunophenotype analysis was performed to characterise the pattern of surface expression of lymphoid and myeloid associated antigens on cell lines and leukaemic blasts used for DNase I hypersensitivity analysis of the CD2 gene. For analysis of cell lines fresh cultured cells were used, whilst for patient derived material or normal controls cryopreserved aliquots of Ficoll separated mononuclear cells were used (see sections 2.6, 2.14) which were defrosted rapidly immediately prior to use. Immunophenotyping was performed in accordance with previously described methods (Ginaldi et al., 1996); all antibodies were used undiluted in the volumes stated below. Cells were washed three times in Hanks medium at room temperature, counted and viability confirmed by trypan blue exclusion; 0.5-1x10^6 cells were used per test. Double
labelling was performed using FITC conjugated CD2 (Caltag Laboratories, Burlingame, CA or Dako, both 5μl) in combination with phycoerythrin (PE) conjugated CD3 (Caltag, 5μl), CD34 (Caltag, 10μl), CD13 (Coulter, 5μl), CD33 (Coulter, 5μl), CD117 (Caltag, 10μl) or CD19 (Caltag, 5μl). Double labelled isotypic mouse immunoglobulins were used as negative controls in all experiments (Caltag, 5μl). These combinations of antibodies were selected to establish CD2 expression in APL samples (CD2/CD34, CD2/CD13, CD2/CD33 and CD2/CD117), determine the degree of T cell contamination in clinical samples used for DNase I hypersensitivity assays (CD2/CD3) and to characterise B lymphoid leukaemias used as negative controls in this assay (CD2/CD19). After addition of the appropriate antibodies to each tube, cells were vortexed, incubated for 10 minutes at room temperature, washed 3 times in phosphate buffered saline (containing 0.02% azide, 0.02% BSA, 0.01% EDTA) and resuspended in 0.5ml isoton prior to analysis by flow cytometry.

2.13.2 Analysis by flow cytometry and CD2 quantification

Flow cytometry was performed using a FACScan (Becton Dickinson) with the Cell Quest software (Becton Dickinson). Data were subsequently analysed after setting a gate on the cell population of interest, as described in Chapter 5. In order to assess the relative expression of CD2 on the cell surface of APL blasts, T lymphoid cell lines, and normal T lymphocytes; quantification was performed using the Quantum Simply Cellular (QSC) Microbeads kit (Sigma, St. Louis, Missouri, USA). This system converts the peak channel reading relating to positive cells into a measure of antibody binding capacity (ABC), thereby providing an indication of the relative number of molecules expressed on the cell surface. The kit includes 4 populations of microbeads, of uniform size equivalent to that of normal lymphocytes, coated with goat-anti-mouse antibodies which differ in their capacity to bind mouse monoclonal antibodies. Also included in the mixture is a blank population of beads with no specific binding capacity for mouse immunoglobulins as a negative control. In order to establish a calibration curve for CD2 expression; 5μl FITC conjugated CD2 antibody (Caltag, Dako) was added to 50μl PBS azide and 100μl QSC beads, vortexed and incubated for 1 hour. The beads were then washed, resuspended in isoton and analysed on the flow cytometer as described above. Peak channel readings from each of the microbead populations were used to construct a standard curve for CD2 expression, from which expression levels on leukaemic cells and normal lymphocytes could be derived using the QSC software program (Sigma). Separate calibration curves were constructed for the two CD2 antibodies used in this
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study (Dako, Caltag) and expression levels of test samples derived from the appropriate curve. For clinical samples, at least 5,000 events were acquired using identical instrument settings for FL1 and FL2 to those employed for microbead analysis. The contribution of Ricardo Morilla (Dept. of Academic Haematology, Royal Marsden Hospital) for the analysis of the flow cytometric data is gratefully acknowledged.

2.14. Tissue culture

Cell lines used for DNase I hypersensitivity assays, PML-immunofluorescence studies and as controls for RT-PCR assays for patients with suspected APL were cultured in RPMI 1640 (ICRF), supplemented with penicillin and streptomycin and 10% fetal calf serum (FCS) in the presence of 5% CO₂ at 37°C. Cell lines were obtained from Cell Services Department, ICRF, Lincoln's Inn Fields, except for the APL cell line NB4 (gift from Michel Lanotte, Hôpital St Louis, Paris) and human erythroleukaemia cell line HEL (ATCC). Aliquots of cells (1 x 10⁷ cells) were routinely stored; cell pellets were resuspended in 1ml Freeze Mix, rapidly transferred to Nunc tubes and placed on ice, tubes were then kept in polystyrene boxes overnight at -70°C, prior to storage in liquid nitrogen. In order to recover the cells, tubes were thawed rapidly, cells were then washed in 15ml RPMI 1640/10% FCS, then resuspended in flasks containing culture medium to a final concentration of 10⁶ cells/ml. Cells were subsequently maintained at 10⁵ cells/ml; sterile procedures were observed throughout.

2.15. Differentiation response of leukaemic cells to ATRA

2.15.1 In vitro differentiation assay

In order to determine the differentiation response of leukaemic blasts to retinoids, ATRA sensitivity assays were performed. Aliquots of NB4 cells or ficoll separated leukaemic blasts derived from patients with AML (10⁶-10⁷ cells) were recovered in RPMI/10% FCS (see section 2.14.). Cells were cultured for 5 days without replenishing the medium, either in the presence of 10⁻⁶M ATRA (Sigma), or with an equal volume of ethanol diluent alone serving as a negative control for the effect of ATRA. Cytospins for subsequent morphological and PML-immunofluorescence assessment were made after recovery of the leukaemic cells and following culture with or without ATRA.
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2.15.2 Nitroblue tetrazolium (NBT) test

In addition to morphological and PML-immunofluorescence assessment, the differentiation response to ATRA was evaluated by determination of the proportion of cells acquiring functional maturity using the NBT (nitroblue tetrazolium) test. This assay measures the respiratory burst, which is important for killing of microbes by phagocytes. Amongst cells of the granulocyte lineage, detection of respiratory burst function is indicative of differentiation to at least the metamyelocyte stage of development. The respiratory burst, which can be potently stimulated by TPA (12-O-tetradecanoylphorbol 13-acetate), is produced by an oxidase system that uses NADPH to reduce oxygen with one electron to superoxide. The generation of superoxide associated with the respiratory burst stimulated by TPA can be detected by the reduction of the yellow NBT to the dark blue insoluble formazan which is clearly visible by light microscopy.

The method used was that previously described by Roberts et al. (1989). Briefly, cells (1 x 10⁶) were incubated for 30 minutes at 37°C in 0.05% (w/v) NBT (Sigma) in RPMI medium containing 1μg/ml TPA (Sigma). Control samples were incubated without TPA in order to determine the resting level of NBT reduction. Samples were then placed on ice for 20 minutes, centrifuged and cell pellets resuspended in 50μl PBS prior to cell counting under the light microscope. In each case the percentage of positive cells containing black deposits was determined after counting a minimum of 200 cells.

2.16. Patient characteristics and treatment

The majority of cases studied were derived from the Medical Research Council Leukaemia trials, particularly the MRC ATRA trial. This latter study sought to determine the optimum means of combining ATRA with chemotherapy for induction therapy of patients with a clinical diagnosis of APL. Patients were randomised to receive ATRA (45mg/m²/day) for 5 days prior to chemotherapy (Short ATRA) or as a more prolonged course commenced with chemotherapy and continued until achievement of complete remission (CR), or to a maximum of 60 days (Extended ATRA). Chemotherapy induction and subsequent consolidation courses were in accordance with the MRC AML 10 protocol for patients <55 years, which was superseded by the AML 12 protocol for patients <60 years; older patients were treated throughout by the AML 11 protocol (see Figures 2.3-2.5). Overall 239 patients were entered into the MRC ATRA trial between January 1993 and January 1997; samples of bone
marrow/peripheral blood taken at or shortly after the time of diagnosis, suitable for molecular analyses were available for 202 patients. Bone marrow samples were also received from a further 6 ATRA trial patients with documented PML/RARα rearrangements (diagnostic investigations documented t(15;17) by cytogenetics, or PML-RARα fusion by FISH) for assessment for minimal residual disease (MRD) by RT-PCR. Trial samples were processed and RNA/DNA extracted at Dept. Haematology, University College Hospital (D.Grimwade 10%, remainder of samples were processed by S.Langabeer and J.Rogers). Central morphological review was provided by Dr D.Swirsky (Royal Postgraduate Medical School). Cytogenetic analyses were provided by 41 local centres in accordance with ISCN guidelines, subject to monitoring by a central quality control assessment scheme (UK, NEQAS), as previously described (Grimwade et al., 1998a). Statistical analyses were performed by K.Wheatley and G.Harrison, Clinical Trial Service Unit, Oxford.

In addition, clinical material was gratefully received, courtesy of Drs P Chipping (Dept. of Haematology, Stoke), S.Kelsey (Royal London Hospital) and M.Neat (St.Bartholemew's Hospital, London), from 2 cases of suspected APL in which the t(15;17) was not identified by conventional cytogenetic analysis and who were not entered into the MRC ATRA trial (section 3.5.1). Molecular analyses were also performed on material derived from 2 patients treated at University College and Middlesex Hospitals, London (courtesy of Drs K.Patterson, A.Kilby and Prof. D.Linch) in whom cytogenetic analyses revealed the t(15;17) in the absence of typical morphological features of APL (section 3.7). Clinical material used for DNase I hypersensitivity assays was derived from cases of APL entered into the MRC trials, samples from patients with other forms of acute or chronic leukaemias were obtained from University College Hospital and St.George's Hospital, London (courtesy of Dr K.Patterson and Dr A.O'Driscoll).
MRC AML 10 protocol for patients < 55 years

**Bone marrow harvest**

DAT 3+10 → DAT 3+8

ADE 10+3+5 → ADE 8+3+5

**MACE**

DAT 3+10
Dauno 50mg/m² d1,3,5
Ara-C 100mg/m² bd d1-10
ThioG 100mg/m² bd d1-10

ADE 10+3+5
Ara-C 100mg/m² bd d1-10
Dauno 50mg/m² d1,3,5
Etop 100mg/m² d1-5

**MidAC**

DAT 3+8
Dauno 50mg/m² d1,3,5
Ara-C 100mg/m² bd d1-8
ThioG 100mg/m² bd d1-8

ADE 8+3+5
Ara-C 100mg/m² bd d1-8
Dauno 50mg/m² d1,3,5
Etop 100mg/m² d1-5

**Abbreviations:**
- Dauno - daunorubicin, Ara-C - cytosine arabinoside, ThioG - 6-thioguanine, Etop - etoposide, m-amsa - m-amsacrine, MTZ - mitozantrone.
MRC AML 12 protocol for patients < 60 years with favourable cytogenetics

Bone marrow harvest

MAE 3+10+5 → MAE 3+8+5
ADE 10+3+5 → ADE 8+3+5

CR

MACE

MAE 3+8+5
ADE 10+3+5 → ADE 8+3+5

NO CR → AML-R protocol

MAE 3+10+5
MTZ 12mg/m² d1,3,5
Ara-C 100mg/m² bd d1-10
Etop 100mg/m² d1-5

MAE 3+8+5
MTZ 12mg/m² d1,3,5
Ara-C 100mg/m² bd d1-8
Etop 100mg/m² d1-5

MACE
m-ems 100mg/m² d1-5
Ara-C 200mg/m² d1-5
Etop 100mg/m² d1-5

MidAC
MTZ 10mg/m² d1-5
Ara-C 1g/m² bd d1-3

ICE
Idarubicin 8mg/m² d1,2
Ara-C 100mg/m² bd d1-3
Etop 100mg/m² d1-3

Figure 2.4
MRC AML 11 protocol for patients > 55 years

DAT 3+10 → DAT 2+5

ADE 10+3+5 → ADE 5+2+5 → DAT 2+7

MAC 3+5 → MAC 2+5

Stop Consol.

COAP → DAT → COAP

α IFN 12mths

NO α IFN

α IFN 12mths

NO α IFN

MAC 3+5
MTZ 12mg/m² d1-3
Ara-C 100mg/m² bd d1-5

COAP
Cyclo 600mg/m² d1
Vincr 1.5mg/m² d1
Ara-C 100mg/m² sc d1-5
Pred 60mg/m² d1-5

α INTERFERON
3 x 10⁶ units 3x/wk

Figure 2.5
Abbreviations: Cyclo - cyclophosphamide,
Vinc - vincristine, Pred - prednisolone
### 2.17 Buffers and solutions

<table>
<thead>
<tr>
<th>Buffer/Solution Description</th>
<th>Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform/isoamyl alcohol</td>
<td>49 parts chloroform to 1 part isoamyl alcohol</td>
</tr>
<tr>
<td>Denhardt's solution (50x)</td>
<td>5g Ficoll (type 400, Pharmacia) 5g polyvinylpyrrolidone, 5g BSA in 500ml distilled water</td>
</tr>
<tr>
<td>DEPC-treated sterile water</td>
<td>0.1% v/v diethyl pyrocarbonate</td>
</tr>
<tr>
<td>Ficoll blue DNA loading buffer (5x)</td>
<td>15% w/v Ficoll (type 400, Pharmacia), 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF</td>
</tr>
<tr>
<td>&quot;Freezing solution&quot; (Freeze mix)</td>
<td>60% RPMI 30% FCS 10% DMSO</td>
</tr>
<tr>
<td>GTC-ME</td>
<td>4M guanidine thiocyanate 0.025 M sodium citrate 0.1M 2-mercaptoethanol 0.5% N-lauryl sarcosine</td>
</tr>
<tr>
<td>IB</td>
<td>10mM NaCl, 5mM MgCl₂, 10mM Tris HCl, pH 7.5</td>
</tr>
<tr>
<td>L- agar</td>
<td>15g agar in 11 LB medium</td>
</tr>
<tr>
<td>LB medium</td>
<td>10g bacto-tryptone 5g bacto-yeast extract 10g NaCl in 11 distilled water, pH 7.0</td>
</tr>
</tbody>
</table>
### Chapter two: Materials and methods

| Oligolabelling buffer (OLB)(5x) | 0.2M Hepes, pH 6.6  
| | 100mM/ml of each of dGTP, dATP, dTTP  
| | 0.25M Tris, pH 8.0  
| | 25mM MgCl₂  
| | 0.35% 2-mercaptoethanol  
| | 25 units/ml random hexamers (Pharmacia)  
| PBSA (11) | 10g NaCl  
| | 0.25g KCl  
| | 0.25g KH₂PO₄  
| | 1.43g Na₂HPO₄  
| Prehybridisation/hybridisation solution: Southern analysis | 5x SSPE  
| | 5x Denhardt's  
| | 0.5% SDS  
| | deionized water  
| | 5mg/ml yeast RNA  
| | (store in 50ml aliquots at 4°C and prewarmed to 65°C before use)  
| Prehybridisation/hybridisation solution: Northern analysis | 5x SSPE  
| | 10x Denhardt's  
| | 100μg/ml sheared salmon sperm DNA  
| | 2% SDS  
| | 50% formamide  
| RNA gel buffer (10x) | 200mM MOPS  
| | 10mM EDTA  
| | 50mM NaOAc, adjust to pH 7 with NaOH  
| | Filter sterilize.  
| RNA loading buffer (10ml) | 1ml 10x RNA gel buffer  
| | 5ml formamide (Fluka)  
| | 1.8ml 35% formaldehyde (Fluka)  
| | 2.2ml sterile water (store)  

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<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
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<tbody>
<tr>
<td>RNA loading dye (10ml)</td>
<td>1.5g ficoll</td>
</tr>
<tr>
<td></td>
<td>0.02g bromophenol blue</td>
</tr>
<tr>
<td>RSB</td>
<td>10mM NaCl,</td>
</tr>
<tr>
<td></td>
<td>3mM MgCl₂,</td>
</tr>
<tr>
<td></td>
<td>10mM Tris HCl, pH 7.5</td>
</tr>
<tr>
<td>20x SSC</td>
<td>175.3g NaCl,</td>
</tr>
<tr>
<td></td>
<td>88.2g sodium citrate in 1l distilled water, pH 7.0</td>
</tr>
<tr>
<td>20x SSPE</td>
<td>175.3g NaCl,</td>
</tr>
<tr>
<td></td>
<td>27.6g NaH₂PO₄,</td>
</tr>
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<td></td>
<td>7.4g EDTA in 1l distilled water, pH 7.4</td>
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<tr>
<td>Stop buffer</td>
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</tr>
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<td>20mM NaCl,</td>
</tr>
<tr>
<td></td>
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</tr>
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</tr>
<tr>
<td></td>
<td>5mM MgCl₂,</td>
</tr>
<tr>
<td></td>
<td>25mM Tris HCl, pH 7.5</td>
</tr>
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<td></td>
<td>- store at 4°C</td>
</tr>
<tr>
<td>50x TAE (1l)</td>
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</tr>
<tr>
<td></td>
<td>57.1ml glacial acetic acid</td>
</tr>
<tr>
<td></td>
<td>100ml 0.5M EDTA</td>
</tr>
<tr>
<td>TBE (1x)</td>
<td>50mM Tris base,</td>
</tr>
<tr>
<td></td>
<td>50mM Boric acid,</td>
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<tr>
<td></td>
<td>2mM EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>10mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>1mM EDTA</td>
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CHAPTER THREE: MOLECULAR CHARACTERISATION OF CASES OF SUSPECTED APL

The presence of the t(15;17) or its molecular consequence, the PML-RARα fusion gene, defines the subgroup of APL cases most likely to gain a beneficial response to retinoids such as ATRA (Miller et al., 1992; Mozziconacci et al., 1998), which has been shown to confer significant improvements in survival when combined with chemotherapy for the treatment of this condition (Fenaux et al., 1994, 1997a; Tallman et al., 1997; Burnett et al., 1997). On this basis, detection of the t(15;17) or an underlying PML/RARα rearrangement in cases of suspected APL is critical not only for optimum patient management but also for meaningful analysis of clinical trials involving such patients. This chapter considers the role of RT-PCR and PML-immunofluorescence techniques as a means of establishing the presence of PML/RARα rearrangements amongst cases entered into the Medical Research Council ATRA trial as compared to conventional cytogenetics (sections 3.1-3.4, 3.6.1). A series of cases of APL which lacked the classic t(15;17) was identified permitting evaluation of the role of PML nuclear body disruption and the relative importance of PML-RARα and RARα-PML fusion products to the pathogenesis of APL (sections 3.5, 3.6.2). Also described are cases of AML with the t(15;17) but lacking typical features of APL, which served to link disruption of PML nuclear bodies and ATRA sensitivity with formation of the PML-RARα fusion gene, irrespective of morphological features of the leukaemic population (section 3.7).

3.1. Establishing the presence of PML/RARα rearrangements using nested RT-PCR

3.1.1 Nested RT-PCR distinguishes 5' from 3' breakpoints in PML

Two major breakpoint cluster regions within PML have been distinguished: 5' breakpoints (bcr 3) typically occur in intron 3, whilst 3' breakpoints disrupt intron 6 (bcr 1) or more proximal exonic or intronic sequence (bcr 2) as indicated on Figures 1.2, 2.2, 3.7 & 3.8. Nested RT-PCR affords a more rapid means of distinguishing PML breakpoint patterns (see Figure 3.1) than Southern blotting. Details of the primers used, relative to the breakpoint regions are provided in Figure 2.2. Previous relatively small studies have suggested that PML breakpoint may be of independent prognostic
RT-PCR DEMONSTRATES 5’ AND 3’ BREAKPOINTS IN PML GENE IN APL

Figure 3.1: RT-PCR demonstrates 5’ and 3’ breakpoints in PML gene in APL. Distinction between APL cases with 5’ (bcr 3) and 3’ (bcr 1/2) PML breakpoints using RT-PCR for amplification of add(15q)-derived PML-RARα and del(17q)-associated RARα-PML transcripts. PML-RARα products have been generated by nested PCR using primers 1 & 8 as external primers, followed by internal primers 2 & 7 (see Figure 2.2). RARα-PML products are generated by primers 5 & 4 followed by 3 & 6. In APL cases with a 3’ PML breakpoint a series of PML-RARα transcripts are generated reflecting alternative splicing between PML central exons. These are represented by PCR products of 426bp (PML exon 3-4-RARα), 685bp (PML exon 3-4-6-RARα) and 829bp (PML exon 3-4-5-6-RARα), as demonstrated by APL patients 14, 19 and APL cell line NB4. In cases with 3’ PML breakpoints that express RARα-PML, a single 144bp product is detected as demonstrated by APL patient 19 and NB4. Cases with a 5’ PML breakpoint (bcr 3) exhibit a single 355bp PML-RARα product (PML exon 3-RARα); however, if the reciprocal is expressed multiple RARα-PML transcripts are detected owing to alternative splicing, as demonstrated by patient 17. In such a situation, RARα-PML products of 215bp (RARα-PML exon 4-7), 474bp (RARα-PML exon 4-6-7) and 618bp (RARα-PML exon 4-5-6-7) may be generated (Borrow et al, 1992). APL patients 13, 15, 16 & 18 were studied in remission for residual disease assessment; in each case disease-related transcripts were not detected. HL60, which lacks a PML/RARα rearrangement was included as a negative RNA control, whilst water controls exclude contamination at RT and PCR steps.
significance (Borrow et al., 1992; Huang et al., 1993; Vahdat et al., 1994) and is also correlated with the presence of additional cytogenetic abnormalities (Slack et al., 1997). This chapter addresses the frequency, clinical correlations and prognostic impact of \( PML \) breakpoint patterns in a large cohort of patients entered into the Medical Research Council ATRA trial.

3.1.2 Validation of nested RT-PCR assay

**Stability of fusion transcripts**

Fresh bone marrow or peripheral blood samples were received from Haematology Centres from across the United Kingdom and Eire. For the majority of samples transit time was less than 2 days; cells were immediately isolated and resuspended in GTC-ME on receipt at University College Hospital, London as described (see section 2.2.1). Fresh material was requested for molecular analysis in preference to RNA or cells suspended in GTC-ME due to concern that employment of different experimental techniques at local centres could limit reliability. Stability of \( PML-RAR\alpha \) and \( RAR\alpha-PML \) fusion transcripts, as well as that of \( PML \) and \( RAR\alpha \) transcripts used as controls for RNA integrity and successful PCR, was established by performing RT-PCR using 1\( \mu \)g total RNA extracted from 1ml aliquots of APL diagnostic bone marrow in heparinised RPMI 1640 taken on sequential days from a sample left on the laboratory bench at room temperature. \( PML, RAR\alpha, PML-RAR\alpha \) and \( RAR\alpha-PML \) transcripts could all be detected in marrow kept at room temperature for at least 6 days, as shown in Figure 3.2. In accordance with this result, RNA of sufficient quality to demonstrate expression of \( PML-RAR\alpha \) transcripts has been extracted from diagnostic specimens in transit for up to 6 days and in a remission sample in transit for at least 4 days. These results are in agreement with previous quantitative RT-PCR results which reported no significant fall in the number of \( PML-RAR\alpha \) transcripts detected in diagnostic bone marrow left at room temperature over a 4 day period (Seale et al., 1997). Quality control assessment of RNA evaluated by gel electrophoresis and success of RT-PCR also established that tubes containing lithium heparin, EDTA or heparinised tissue culture medium are all suitable for transport of specimens to the laboratory.
STABILITY OF PML-RARα & RARα-PML mRNA FUSION TRANSCRIPTS

Figure 3.2

Stability of PML-RARα and RARα-PML mRNA was determined by performing RT-PCR using 1μg total RNA extracted from 1ml aliquots of APL diagnostic bone marrow taken on sequential days from a sample left on the laboratory bench at room temperature. The patient was found to have a 3' PML breakpoint pattern. PML-RARα and RARα-PML transcripts could be detected for at least 6 days from the time of marrow aspiration. Also shown are HL60 and NB4 cell line controls, together with water controls to exclude contamination at the RT and PCR steps. PML and RARα transcripts were also detectable over the 6 day period (data not shown).
Chapter three: Molecular characterisation of cases of suspected APL

Sensitivity

To establish the sensitivity of the assay, nested RT-PCR was performed on total RNA derived from a series of 10-fold dilutions of the APL cell line NB4 (bcr 1 PML breakpoint, RARα-PML positive) in the AML M2 cell line HL60 serving as filler cells which do not carry a PML/RARα rearrangement. The total cell count for each dilution was 1 x 10^7 cells, from which RNA was extracted and 1μg used for cDNA synthesis for subsequent PCR reactions. Primer sets to detect PML-RARα could detect 1 APL cell in 10^4 HL60 cells, whereas RARα-PML was routinely detected at a greater sensitivity of 1 in 10^5, see Figure 3.3. Due to lack of availability of an APL cell line with a 5' (bcr 3) PML breakpoint, the sensitivity of PML-RARα and RARα-PML assays for cases with this breakpoint pattern was evaluated using serially diluted cDNA derived from 1μg total RNA from a sample of diagnostic bone marrow. PML-RARα transcripts were detected to a dilution of 1 in 10^4, whereas in accordance with the results obtained with the NB4 cell line RARα-PML transcripts were detected to a dilution of 1 in 10^5 (see Figure 3.4). At dilutions of 1 in 10^4 and 10^5, only the 618bp RARα-PML transcript was detected; subsequent sequence analysis confirmed that this product corresponded to fusion of RARα to PML exon 4 and included PML exons 5, 6 and 7. Taking into account the assessment of the stability of fusion transcripts resulting from the t(15;17) described above, the increased sensitivity of the RARα-PML assay which is observed for both breakpoint patterns irrespective of alternative splicing may be indicative of a higher level of expression of RARα-PML compared with PML-RARα.
Sensitivity of *PML-RARα* and *RARα-PML* nested RT-PCR assays: 3' *PML* breakpoint

---

**Figure 3.3**

PCR sensitivity assay. RT-PCR was performed using 1μg total RNA derived from serial 10-fold dilutions of NB4 cell line in a background of HL60 AML M2 cell line, serving as *PML-RARα/RARα-PML* negative filler cells. NB4 demonstrates a 3' (bcr 1) *PML* breakpoint pattern with expression of *RARα-PML*. Primer sets for *PML-RARα* detected 1 NB4 cell at a dilution of 1 in 10⁴ HL60; whereas *RARα-PML* was detected at a sensitivity of 1 in 10⁵. Also shown is corresponding amplification of *PML* transcripts as a control for RNA integrity; multiple bands reflect alternative splicing between *PML* central exons. Water controls were included to exclude contamination at RT and PCR steps.
Sensitivity of \textit{PML-RAR}\textsubscript{\alpha} and \textit{RAR}\textsubscript{\alpha}-\textit{PML} nested RT-PCR assays: 5' \textit{PML} breakpoint

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.4.png}
\caption{Determination of sensitivity of \textit{PML-RAR}\textsubscript{\alpha} and \textit{RAR}\textsubscript{\alpha}-\textit{PML} assays in APL associated with 5' breakpoints in \textit{PML}. In the left hand six lanes are shown RT-PCR analyses of serial dilutions of cDNA derived from 1\,\mu g total RNA extracted from a sample of diagnostic bone marrow. \textit{PML-RAR}\textsubscript{\alpha} transcripts were detected to a dilution of 1 in 10\textsuperscript{4}, whereas in accordance with the results obtained with the NB4 cell line \textit{RAR}\textsubscript{\alpha}-\textit{PML} transcripts were detected to a dilution of 1 in 10\textsuperscript{5} (see Figure 3.3). At dilutions of 1 in 10\textsuperscript{4} and 10\textsuperscript{5}, only the 618bp \textit{RAR}\textsubscript{\alpha}-\textit{PML} transcript was detected; subsequent sequence analysis confirmed that this band corresponded to fusion of \textit{RAR}\textsubscript{\alpha} to \textit{PML} exon 4 and included \textit{PML} exons 5, 6 and 7.}
\end{figure}
Chapter three: Molecular characterisation of cases of suspected APL

Participation in International quality control schemes
RT-PCR methods used for establishing the presence of \( PML/RAR\alpha \) rearrangements in APL in this study were validated by results obtained in two independent external European Quality Control Schemes, coordinated by Prof. Christine Chomienne (Hôpital St. Louis, Paris) and Dr Pascual Bolufer Gilabert (Hospital Universitario La Fe, Valencia), respectively. In both schemes coded samples were received for analysis and participants later informed as to their performance.

The French scheme provided 3 samples of cells suspended in GTC-ME (labelled in red, black and blue, corresponding to lanes 1, 2 and 3 in Figure 3.5A, respectively), thereby permitting evaluation of RNA synthesis as well as all stages of the RT-PCR technique. Satisfactory RNA extraction from each of the samples was indicated by successful amplification of \( PML \) and \( RAR\alpha \) transcripts as shown in Figure 3.5A. \( PML-RAR\alpha \) and \( RAR\alpha-PML \) fusion transcripts with a 3' \( PML \) breakpoint pattern were amplified from samples labelled in black and blue (later revealed to be \( 1 \times 10^7 \) (blue) and \( 2 \times 10^6 \) (black) NB4 cells as positive controls), whilst no fusion transcripts were amplified from the sample labelled in red (later revealed to be \( 1 \times 10^7 \) HL60 negative control).

The Spanish scheme provided 4 cDNA samples (labelled 10-13) and 1 RNA sample (labelled 14) for analysis. No \( PML \) or \( RAR\alpha \) transcripts were amplified from samples 12 and 13 (see Figure 3.5B), indicating degradation of the cDNA or failure at a step prior to cDNA synthesis and these samples were therefore not considered suitable for further analysis. \( PML \) and \( RAR\alpha \) transcripts were successfully amplified from the remaining samples received. \( PML-RAR\alpha \) and \( RAR\alpha-PML \) transcripts with a 3' \( PML \) breakpoint pattern were detected in samples 10 and 14, whilst no fusion transcripts were amplified from case 11 as shown in Figure 3.5B. Sample 10 was derived from an APL patient considered by the majority of quality control scheme participants to have a 3' \( PML \) breakpoint, cDNA 11 was revealed to be a negative control derived from a case of CML, whilst RNA 14 was a positive control derived from a 1/5 dilution of NB4 in non APL RNA.
Figure 3.5: RT-PCR analysis of material received from European quality control schemes. Panels A and B refer to samples received from France and Spain, respectively. Details of coded test samples are provided in section 3.1.2. As before, 1/1000 dilution NB4, HL60 and water controls were also run in parallel. Also shown in panel B are analyses of RNA derived from a non-APL patient and from a case of APL with a bcr 3 PML breakpoint.
Chapter three: Molecular characterisation of cases of suspected APL

3.2. Frequency of \textit{PML/RAR}α rearrangements in cases of suspected APL entered into the MRC ATRA trial - as determined by RT-PCR

Overall 239 patients considered by referring clinicians to have APL were entered into the MRC ATRA trial. Details of the cytogenetic and molecular characterisation of these patients is outlined in Figure 3.6. Clinical material taken at the time of diagnosis, or shortly thereafter, was received for molecular analysis from 202/239 cases. Overall, RT-PCR confirmed the clinical diagnosis of APL in 187/202 (93\%) patients; in 186 cases underlying \textit{PML/RAR}α rearrangements were identified, whilst in the remaining case APL was associated with a t(11;17)(q23;q12-21) abnormality leading to a \textit{PLZF/RAR}α rearrangement (see section 3.5.2). Expression of reciprocal del(17q) derived \textit{RAR}α-\textit{PML} transcripts was detected in 142/186 (76\%) patients with \textit{PML/RAR}α rearrangements, and in 8 patients \textit{RAR}α-\textit{PML} was the sole fusion transcript detected. In 4 of these cases (each with an identified t(15;17)), RT-PCR analysis was performed using bone marrow taken after induction therapy and absence of \textit{PML-RAR}α transcripts most likely reflected the relative insensitivity of this assay, compared to that for \textit{RAR}α-\textit{PML}. In support of this view, both fusion transcripts were detected in 1 of these patients studied at the time of subsequent relapse. Failure to detect \textit{PML-RAR}α fusion transcripts in the remaining 4 cases could have reflected relatively poor RNA quality despite satisfactory \textit{PML} and \textit{RAR}α controls, since in 3 of the patients cytogenetic analysis identified an underlying t(15;17) including one case in which formation of the \textit{PML-RAR}α fusion gene was confirmed by FISH analysis using \textit{PML} 15.5 and \textit{RAR}α 121 cosmid probes (performed by P. Gorman, ICRF).

RT-PCR was found to significantly enhance the detection of 15;17 rearrangements amongst ATRA trial patients compared with conventional cytogenetics (see Figure 3.6). All of the cases with documented t(15;17) (n=155) or with 3-way translocations involving chromosomes 15 and 17 (n=3) from whom material was received for RT-PCR analysis were confirmed to have \textit{PML/RAR}α rearrangements. In addition, a further 28 patients with APL in whom the t(15;17) was not identified by conventional cytogenetics were found to have underlying \textit{PML/RAR}α rearrangements by RT-PCR. Cytogenetic analyses in this latter group were most commonly normal (n=12) or failed (n=10); in the remaining 6 patients other clonal abnormalities were detected of which 2 had chromosomes 15 and 17 of normal appearance. Amongst the 12 patients with normal karyotype and documented \textit{PML/RAR}α rearrangements, \textit{PML-RAR}α and \textit{RAR}α-\textit{PML} fusion transcripts were both detected in 6 suggesting that the conventional t(15;17) had occurred, but had been missed despite culture of marrow cells for at least 16 hours prior
Molecular and cytogenetic characterisation of patients entered into the MRC ATRA trial.

Results of RT-PCR and PML-immunofluorescence analyses are described in this thesis. FISH analyses were performed by S.Iqbal and are described elsewhere (Iqbal et al, 1998). Morphology review was performed by D.Swirsky.
Chapter three: Molecular characterisation of cases of suspected APL

to analysis. In the remaining 6 patients PML-RARα was the sole fusion transcript detected consistent with insertion events; further characterisation of a series of such cases using FISH is described below (section 3.5.1).

In 15/202 cases derived from the MRC ATRA trial, molecular screening for underlying PML/RARα and PLZF/RARα rearrangements by RT-PCR was negative and indeed subsequent morphologic review (performed by Dr D. Swirsky, Royal Postgraduate Medical School) did not support the original clinical diagnosis of APL. Indeed 4 of these cases subjected to molecular screening were found to have primary cytogenetic abnormalities associated with other subtypes of AML (t(8;21), n=3; inv(16), n=1). Absence of underlying PML/RARα rearrangements was supported by the results of the PML-immunofluorescence assay in each of the 9 cases with suitable material for analysis (see section 3.6.1 and Figure 3.17). Furthermore, the RT-PCR results were subsequently confirmed by interphase FISH in each of the 6 patients analysed using locus specific probes for the PML-RARα fusion, performed by S. Iqbal (RPMS) and described elsewhere (Iqbal et al., 1998).

3.3. Determination of PML breakpoints

3.3.1 Distribution of PML breakpoints amongst MRC ATRA trial patients with PML/RARα rearrangements

5' (bcr 3) and 3' (bcr 1/2) PML breakpoints were distinguished by RT-PCR; results were consistent with a 5' breakpoint in 72/186 (39%) and a 3' breakpoint in 114/186 (61%). Breakpoints within the 3' region of PML were further characterised in 100 cases, distinguishing disruption within intron 6 (bcr 1) from involvement of more proximal regions of the gene (bcr 2). For the majority of patients with 3' PML breakpoints, nested RT-PCR using a PML exon 3 internal primer (primer 2, Figure 2.2) yields two PML-RARα fragments containing PML exon 6 (829bp and 685bp) which differ in relation to the presence of exon 5 due to alternative splicing. For cases found to have 3' PML breakpoints, the second round of PCR was routinely repeated using a PML exon 5 primer (primer 9, Figure 2.2), thereby eliminating the effects of alternative splicing and generating single band products suitable for subsequent sequence analysis (see Figure 3.7, panel c). All PCR products generated by both sets of second round primers were analysed by Southern hybridisation to an oligonucleotide probe specific for the PML exon 6-RARα junction (5'-TCTCAATGGCTGCTC-3'), designed to detect breakpoints
Figure 3.7: Distinction of bcr 1 and bcr 2, 3' breakpoints in PML using a PML-RARα junctional oligonucleotide probe.

Panel a: PML-RARα RT-PCR products generated in APL patients with 5' and 3' PML breakpoints using primers 1 & 8 as external primers, followed by nested primers 2 & 7 (see Figure 2.2). Panel e: Distinction of bcr 2 from bcr 1 3' PML breakpoints by RT-PCR using a PML exon 5 primer (primer 9, Figure 2.2) for the second round of amplification. Product of identical size to that generated by NB4 cDNA was consistent with a bcr 1 breakpoint in APL cases loaded to lanes 5-7; whilst a product size differing from NB4 was indicative of a bcr 2 breakpoint in lane 8. However, this approach failed to identify a less common PML exon 5 bcr 2 breakpoint as shown by the case in lane 4, in which no product was amplified by the second round PCR in common with bcr 3 breakpoints (lane 3). Panel b/d: Hybridisation of PCR products shown in panel a and c respectively to an oligonucleotide probe spanning the PML exon 6-RARα 3' cDNA junction, confirming presence of bcr 1 breakpoints in APL cases in lanes 5-7 and the NB4 cell line. Absence of hybridisation in lane 8 reflected the presence of a bcr 2 breakpoint, confirmed by subsequent sequence analysis. Panel e: Detection of del(17q)-derived RARα-PML transcripts. In APL cases loaded to lanes 1 & 2, RARα PML transcripts were detected in the absence of PML-RARα. The presence of a 144bp product in isolation in lane 1 was consistent with a 3' PML breakpoint, but did not permit distinction between bcr 1 & 2 breakpoints. A 215bp product in lane 2 was indicative of a bcr 3 breakpoint.
within the bcr 1 region. Lack of hybridisation to the oligonucleotide probe indicated the occurrence of a bcr 2 breakpoint (see Figure 3.7, panel d). Initially, the reliability of junctional oligonucleotide probe hybridisation to distinguish bcr 1 and bcr 2 PML breakpoints was established by evaluation in 50 cases, which were also investigated by sequence analysis of PCR products (performed in both directions using appropriate internal primers). Both techniques yielded identical results, identifying 5 bcr 2 cases. A further 50 cases with 3' PML breakpoints were screened by oligonucleotide probe hybridisation, revealing 7 bcr 2 cases; subsequent sequence analysis was successful in 4 of these cases and confirmed disruption of PML within the bcr 2 region as described below.

3.3.2 Characterisation of APL cases with PML breakpoints within the bcr 2 region

Overall 10 bcr 2 cases (b1-10) were further characterised by sequence analysis of PML-RARα RT-PCR products (see Figures 3.8, 3.9); 9 were derived from the ATRA trial as mentioned above, whereas the remaining patient (b8) was not entered into the trial and is described in more detail below (sections 3.7.1, 3.7.2). In 9/10 cases disruption of PML occurred in exon 6, whilst in the remaining case (b1) the breakpoint involved exon 5 which has not been reported previously (Figure 3.8). The PML-RARα reading frame was invariably maintained and in 7 cases this was associated with the inclusion of 7-49 nucleotides between PML and RARα exonic sequences (Figure 3.9). A previous study characterising the genomic breakpoint region of an APL bcr 2 case, demonstrated that the intervening nucleotides were derived from RARα intron 2 situated immediately 5' to a cryptic splice donor consensus site (Pandolfi et al, 1992). Similarly, the additional nucleotides associated with case b5 were found to be derived from RARα intron 2 close to the site of a previously described translocation breakpoint (Yoshida et al, 1995). Published primers (forward: 5'-CACCAGTCTGGATTGTCTAT-3'; reverse 5'-TGACTATGTGCTACCACCCCT-3') flanking the predicted breakpoint were used to amplify this segment of intronic sequence by PCR using DNA from RARα cosmid 121, which encompasses the entire APL breakpoint region, as template. The resultant PCR product was sequenced and confirmed to contain the stretch of additional nucleotides detected in the PML-RARα fusion cDNA from case b5, which was also situated immediately 5' to a cryptic splice donor consensus site (see Figure 3.10 panel B), in agreement with the case characterised by Pandolfi and colleagues (Pandolfi et al., 1992). Interestingly, in 2 cases (b4 & b9) with different PML breakpoints, the 45bp stretch of additional nucleotides was found to be identical (see Figure 3.9). Furthermore, the
CHARACTERISATION OF APL CASES WITH bcr 2 PML BREAKPOINTS

Figure 3.8

Position of bcr 2 breakpoints identified in 10 patients (numbered b1-b10), relative to PML cDNA sequence (Goddard et al, 1991; Fagioli et al, 1992). Exon boundaries are denoted by vertical bars. Three patients (b6-8) shared an identical PML breakpoint, adjacent to a cryptic splice donor consensus (underlined). Sequence analysis of the PML-RARα fusion transcripts detected in these patients is presented in Figure 3.9.
CHARACTERISATION OF APL CASES WITH bcr 2 PML BREAKPOINTS

Figure 3.9

Junctional PML-RARα cDNA sequences delineated in 10 patients (b1-b10) with disruption of PML within the bcr 2 region, corresponding to breakpoints shown in Figure 3.8. PML and RARα exonic sequences are shaded in light and darker grey, respectively. Intervening nucleotides are unshaded; in 3 cases (b2, b4 & b9) these were found to be homologous (underlined). Cases b4 & b9 were noted to have an identical stretch of 45bp in the fusion cDNA separating PML & RARα exonic sequences, whilst in case b2 only 7 intervening nucleotides were present, rendering the significance of the homology less certain. In 3 cases (b6-8), in which the PML breakpoint was immediately adjacent to a cryptic splice donor site (Figure 3.8), PML and RARα exonic sequences were directly apposed in the PML-RARα fusion cDNA.
For APL cases with bcr2 PML breakpoints immediately adjacent to cryptic splice donor consensus sites (e.g. cases b6-b8, shown in Figures 3.8 and 3.9), PML-RARα cDNA was generated through splicing of PML exon 6 directly to RARα exon 3, as shown in panel A. In the remaining bcr2 cases the PML breakpoint was not immediately adjacent to such a site (see Figure 3.8). Sequence analysis of RARα in the breakpoint region of patient b5, confirmed that in such cases a cryptic donor consensus site is derived from RARα intron 2. Hence, the intervening junctional nucleotides detected in bcr2 PML-RARα fusion cDNAs (e.g. cases b1-5, b9,10, Figure 3.9) are derived from RARα intron 2, lying between the break/fusion site and the next available cryptic splice donor consensus site, thereby permitting splicing of PML exon 6 (exon 5, case b1) and the additional nucleotides to RARα exon 3 as shown in panel B.
additional sequence was homologous over its 3’ region with the 7bp insert identified in case b2 and a 29bp insert from a previously characterised bcr 2 case (Pandolfi et al., 1992), with an identical boundary between the insert and RARα exonic sequences in the PML-RARα fusion cDNA. This indicates the presence of a recombination hotspot within RARα intron 2 (which extends for at least 10 kb), which is presumably adjacent to a suitable cryptic splice donor site.

In 3 patients (b6-8) with bcr 2 breakpoints, sequence analysis of PML-RARα fusion cDNA revealed direct apposition of PML and RARα sequences (Figure 3.9). In these cases the PML breakpoint was found to be adjacent to the same cryptic splice donor consensus site, as shown in Figures 3.8 and 3.10. This breakpoint appears to be relatively common accounting for 5/14 previously characterised bcr 2 cases (Pandolfi et al., 1992; Gallagher et al., 1995).

For bcr 2 APL cases, this clustering of breakpoints within PML and also within RARα, as evidenced by the frequent inclusion of identical additional nucleotides in the fusion cDNA, could reflect the satisfaction of a number of conditions in order to generate a translocation product of leukaemogenic potential. In particular, this appears dependent on the acquisition of a suitable splice donor consensus site, maintenance of the reading frame, with relatively few nucleotides separating PML and RARα exonic sequence. As to whether secondary DNA structure or particular sequence motifs (e.g. topoisomerase II sites) within PML or RARα intron 2 play an important role in the generation of the t(15;17) remain to be determined.

3.4 Correlation between molecular characteristics, disease parameters and outcome amongst patients entered into the MRC ATRA trial

No correlation was observed between PML breakpoint pattern and expression of RARα-PML, presenting leucocyte count (WBC), presence of additional cytogenetic abnormalities or achievement of complete remission (CR) (Table 3.1). There was a trend towards an increased risk of relapse amongst patients with 5’ PML breakpoints (Figure 3.11), although this did not translate into any difference in overall survival (Figure 3.12). Expression of RARα-PML transcripts was found to have no significant impact on a variety of disease characteristics including presenting WBC, presence of additional cytogenetic abnormalities or prognosis (see Table 3.1). Overall, patients in whom underlying PML/RARα rearrangements were identified solely by molecular techniques
<table>
<thead>
<tr>
<th>PML bkpt/ expression of RARα-PML</th>
<th>Total (n=186)</th>
<th>Cytogenetics: t(15;17) alone (n=96)</th>
<th>Cytogenetics: t(15;17) with additional changes (n=62)</th>
<th>Cytogenetics: no t(15;17), PML/RARα rearrangement by RT-PCR (n=28)</th>
<th>Age (median)</th>
<th>WBC (10⁹/l)</th>
<th>CR rate</th>
<th>Relapse Risk (4 yrs)</th>
<th>Overall Survival (4 yrs)</th>
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<tbody>
<tr>
<td>5' (bcr 3)</td>
<td>72 (39%)</td>
<td>37 (39%)</td>
<td>23 (37%)</td>
<td>12 (43%)</td>
<td>42.5 (3-69)</td>
<td>2.9 (0.2-195)</td>
<td>81%</td>
<td>38%</td>
<td>59%</td>
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<tr>
<td>3' (bcr 1/2*)</td>
<td>114 (61%)</td>
<td>59 (61%)</td>
<td>39 (63%)</td>
<td>16 (57%)</td>
<td>38.5 (1-73)</td>
<td>2.2 (0.3-99)</td>
<td>82%</td>
<td>21%</td>
<td>67%</td>
</tr>
<tr>
<td>RARα-PML +ve</td>
<td>142 (76%)</td>
<td>79 (82%)</td>
<td>50 (81%)</td>
<td>13 (46%)</td>
<td>40 (1-72)</td>
<td>2.6 (0.2-195)</td>
<td>80%</td>
<td>29%</td>
<td>63%</td>
</tr>
<tr>
<td>5'</td>
<td>57 (79%)</td>
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<td>19 (83%)</td>
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<td>6 (50%)</td>
<td>43 (3-69)</td>
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<td>47 (80%)</td>
<td>3'</td>
<td>31 (79%)</td>
<td>3'</td>
<td>7 (44%)</td>
<td>40 (1-72)</td>
<td>2.4 (0.3-99)</td>
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<tr>
<td>RARα-PML -ve</td>
<td>44 (24%)</td>
<td>17 (18%)</td>
<td>12 (19%)</td>
<td>15 (54%)</td>
<td>38 (4-73)</td>
<td>1.9 (0.5-144)</td>
<td>84%</td>
<td>22%</td>
<td>66%</td>
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</table>

Table 3.1
Relationship between PML breakpoint, expression of RARα-PML and disease characteristics, in MRC ATRA trial patients.
Figure 3.11: MRC ATRA trial – Relapse-free survival by PML breakpoint

At risk:

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<td>93</td>
<td>58</td>
</tr>
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<td>1</td>
<td>78</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
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</tr>
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No. Patients

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<td>58</td>
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<tr>
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<td>17</td>
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No. Events

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<td>5'</td>
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</table>

\[ P = 0.1 \]

% still relapse-free
Figure 3.12: MRC ATRA trial – Survival by PML breakpoint

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<th>No. Patients</th>
<th>No. Events</th>
<th>2P = 0.4</th>
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<td></td>
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<td>37</td>
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<td>24.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.4</td>
</tr>
</tbody>
</table>

% still alive

67%

59%
Figure 3.13: MRC ATRA trial – Survival by t(15;17)

% still alive

0 25 50 75 100

Years from entry

At risk:
cytogenetic molecular none
187 28 18
130 23 11
104 17 8
82 10 4
48 6 2

No. Patients | No. Events
--- | ---
cytogenetic molecular none
187 | 72 | 69.7
28 | 6 | 11.0
18 | 9 | 6.3

2P = 0.9
were found to share the relatively favourable prognosis of those with documented t(15;17) (see Figure 3.13). This result is consistent with both groups indeed having the same disease and highlights the importance of molecular screening for appropriate treatment of patients with suspected APL and meaningful analysis of clinical trials involving such patients.

The \textit{PML} breakpoint frequency observed in this study is similar to that in other recently published series (Mandelli \textit{et al}., 1997, Gallagher \textit{et al}., 1997); however, no relationship was found between breakpoint and additional cytogenetic abnormalities as reported in a previous much smaller study (Slack \textit{et al}., 1997). Whether \textit{PML} breakpoint influences outcome is a somewhat contentious issue (Vahdat \textit{et al}., 1994; Fukutani \textit{et al}., 1995; Gallagher \textit{et al}., 1997), probably reflecting relatively small sample sizes and inter- and intra- study treatment variation. A relatively small study (n=45) from Memorial Sloan Kettering (Vahdat \textit{et al}., 1994), suggesting that the presence of a 5' \textit{(bcr 3)} \textit{PML} breakpoint is associated with an increased risk of relapse. This is in accordance with the trend observed in the MRC ATRA trial; however since no difference in overall survival was observed, this would suggest that there is insufficient data at present to justify use of \textit{PML} breakpoint pattern as a means of directing treatment approach in individual patients. Indeed, other groups have observed no difference in outcome according to \textit{PML} breakpoint (Fukutani \textit{et al}., 1995; Gallagher \textit{et al}., 1997), suggesting that any such effect may reflect therapeutic differences between the studies, influence of presenting WBC or merely the play of chance.

3.5. Molecular characterisation of APL cases lacking the t(15;17)

Availability of suitable material for molecular and FISH analyses permitted further investigation of the mechanisms underlying APL in 7 cases found to lack the classic t(15;17) by conventional cytogenetics. Details of the morphologic, cytogenetic and molecular findings in these cases are presented below and summarised in Table 3.2. Five were derived from the MRC ATRA trial, whilst the remaining patients (cases 1 & 3) were treated in accordance with alternative protocols.
<table>
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<th>Case</th>
<th>Morphological features</th>
<th>Cytogenetics</th>
<th>Fusion transcripts detected by RT-PCR</th>
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<tr>
<td>C 2</td>
<td>Classical hypergranular M3 Numerous Auer rods</td>
<td>46,XY</td>
<td>PML-RARα</td>
<td>bcr 1</td>
<td>Interstitial insertion of RARα into PML on 15q</td>
<td>microparticulate</td>
</tr>
<tr>
<td>C 3</td>
<td>Hypogranular M3 variant</td>
<td>46,XY</td>
<td>PML-RARα</td>
<td>bcr 3</td>
<td>Interstitial insertion of RARα into PML on 15q</td>
<td>N/D</td>
</tr>
<tr>
<td>C 4</td>
<td>Classical hypergranular M3 Numerous Auer rods</td>
<td>46,XY,t(4;16)(q14;q22),t(9;12)(q22;q24), dir ins(17;15)(q21;q15q22) [11] 46,idem,t(6;8)(q13;q22) [10] 46,idem,add(3q),t(6;14),t(11;22) [3] 46,XY [6]</td>
<td>PML-RARα</td>
<td>bcr 1</td>
<td>Interstitial insertion of PML into RARα on 17q</td>
<td>N/D</td>
</tr>
<tr>
<td>C 5</td>
<td>Classical hypergranular M3 Numerous Auer rods</td>
<td>46,XX,del(7)(q22q36) [5] 46,XX [5]</td>
<td>PML-RARα</td>
<td>bcr 3</td>
<td>PML-RARα fusion detected by interphase FISH</td>
<td>microparticulate</td>
</tr>
<tr>
<td>C 6</td>
<td>Classical hypergranular M3 Numerous Auer rods</td>
<td>46,XX</td>
<td>PML-RARα</td>
<td>bcr 3</td>
<td>PML-RARα fusion detected by interphase FISH</td>
<td>microparticulate</td>
</tr>
<tr>
<td>C 7</td>
<td>Hypergranular M3 Marrow replaced by abnormal promyelocytes with basophilic granules. No Auer rods observed</td>
<td>46,XY,t(11;17)(q23;q12-21) [12] 46,XY [3]</td>
<td>PLZF-RARα, RARα-PLZF</td>
<td>N/A</td>
<td>N/D</td>
<td>wild-type</td>
</tr>
</tbody>
</table>

Table 3.2 Molecular and cytogenetic characteristics of patients with morphologic APL, lacking the t(15;17) N/D: not determined, N/A: not applicable
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3.5.1 Cryptic PML/RAR\(\alpha\) rearrangements

RT-PCR identified an underlying PML/RAR\(\alpha\) rearrangement in 6/7 patients (Cases 1-6, Table 3.2). In each case PML-RAR\(\alpha\) transcripts were detected in the absence of RAR\(\alpha\)-PML, consistent with the concept that PML-RAR\(\alpha\) is the critical oncogenic fusion protein. Formation of the PML-RAR\(\alpha\) fusion in these cases was subsequently confirmed by FISH analyses (performed by P. Gorman, ICRF) using ICRF probes PML cos 15.5 and RAR\(\alpha\) cos 121, prepared as described in section 2.1.1. In 4 patients with evaluable metaphase spreads, analyses using the ICRF cosmid probes which specifically detect the PML-RAR\(\alpha\) fusion, were complemented by FISH studies using commercial PML and RAR\(\alpha\) probes (Oncor, Gaithersburg, MD) which optimally detect RAR\(\alpha\)-PML, together with centromere probes (Oncor, Gaithersburg, MD) and whole chromosome paints (WCPs; Vysis, Richmond, Surrey) for chromosomes 15 and 17. In each case results were consistent with the occurrence of a non-reciprocal translocation with sole formation of the PML-RAR\(\alpha\) fusion gene. In 3 patients, in each of whom morphologically normal chromosomes 15 and 17 were identified by conventional cytogenetics (patients 1-3, Table 3.2), the PML-RAR\(\alpha\) fusion gene was localised to chromosome 15q, (e.g. case 2 shown in Figure 3.14a,b). In each of these cases, 15 and 17 specific paints hybridised solely to their respective chromosomes (see Figure 3.14c,d) consistent with an interstitial insertion of RAR\(\alpha\) into PML on 15q. Furthermore in each case, using commercially available probes (Oncor), RAR\(\alpha\) was found to hybridise to two normal appearing chromosome 17s in addition to forming a fusion signal on 15q; again consistent with formation of a PML-RAR\(\alpha\) fusion on 15q and absence of the reciprocal RAR\(\alpha\)-PML fusion gene as suggested by RT-PCR analyses. In the remaining patient with evaluable metaphases PML 15.5 and RAR\(\alpha\) 121 probes localised the PML-RAR\(\alpha\) fusion to 17q (case 4, Table 3.2; see Figure 3.15a,b). In addition to the PML-RAR\(\alpha\) fusion signal, a more centromeric RAR\(\alpha\) hybridisation signal was observed on 17q. WCPs demonstrated insertion of chromosome 15 material into 17q, such that chromosome 17 appeared abnormally large on conventional cytogenetic assessment (see Figure 3.15c,d). These results indicate that the PML-RAR\(\alpha\) fusion in this patient reflected insertion of PML with more centromeric chromosome 15 derived-material into the genomic region spanned by RAR\(\alpha\) cos 121; again this was consistent with detection of PML-RAR\(\alpha\) in the absence of RAR\(\alpha\)-PML fusion transcripts by RT-PCR.

All FISH analyses using locus-specific probes were performed and reported in a "single-blinded" fashion. In addition to APL cases with cryptic PML/RAR\(\alpha\) rearrangements, a series of control samples were studied using ICRF and Oncor locus specific probes.
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Figure 3.14 Cryptic PML-RARα fusion resulting from insertion of RARα into PML on 15q (case no. 2, Table 3.2)

(a) FISH analysis using ICRF PML 15.5 (green) and RARα 121 (red) cosmid probes. The RARα probe hybridised to two chromosome 17s of normal appearance, whereas 1 normal PML locus was observed on chromosome 15. The PML-RARα fusion was detected on 15q (yellow arrow). Localisation of the fusion gene was confirmed by subsequent hybridisation with a chromosome 15 centromere probe, shown in red in (b).
(c) Chromosome 15 paint (red); chromosome 17 centromere probe (green).
(d) Chromosome 17 paint (green); chromosome 15 centromere probe (red).
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**Figure 3.15** PML-RARα fusion resulting from insertion of PML with associated chromosome 15-derived material into RARα on 17q (case no. 4, Table 3.2).

(a) FISH analysis using ICRF PML (green) and RARα (red) cosmid probes. The PML-RARα fusion gene was detected on 17q (yellow arrow), adjacent to RARα hybridisation signal (red arrow). Splitting of RARα-derived signal on der(17q) was indicative of insertion of PML and adjacent sequence into the genomic region covered by RARα cosmid 121. Localisation of the PML-RARα fusion gene was confirmed by subsequent hybridisation with a chromosome 17 centromere probe shown in green in (b). (c) Chromosome 15 paint (red) and chromosome 17 centromere probe (green), confirming insertion of chromosome 15-derived material into 17q. (d) Chromosome 17 paint (green), chromosome 15 centromere probe (red). Chromosome 17 paint remained localised to 17; der(17q) showed a region of absent signal corresponding to the inserted region of 15q shown in (c).
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*PML/RARα* rearrangements were excluded in 3/3 patients with other subtypes of AML used as negative controls; whereas their presence was confirmed in 8/8 patients with documented t(15;17). This latter group included 2 cases in which RT-PCR had detected a single fusion transcript. In the patient in which *RARα-PML* transcripts were the sole fusion species detected by RT-PCR, FISH using ICRF cosmid probes detected a *PML-RARα* fusion signal on add(15q); thereby suggesting that apparent absence of *PML-RARα* fusion transcripts in this case was indicative of PCR failure rather than the occurrence of a non-reciprocal translocation (qv section 3.2); although it remains a possibility that *PML-RARα* was not expressed. Similarly, in the patient with the t(15;17) in which *PML-RARα* was the sole fusion transcript detectable, a fusion signal was localised to del(17q) using Oncor *PML* and *RARα* probes. In this case a bcr 1 *PML* breakpoint was identified; since the *RARα-PML* RT-PCR is more sensitive than that for *PML-RARα* in patients with this breakpoint (section 3.1.2), the findings in this patient would suggest that in some APL cases, the reciprocal *RARα-PML* fusion gene is formed but not expressed.

3.5.2 t(11;17) associated APL

In 1 patient with morphologically confirmed APL derived from the MRC ATRA trial, cytogenetic analysis revealed t(11;17)(q23;q12-21) (Case 7, Table 3.2). Details of the clinical and morphological features of this case have been presented elsewhere (Culligan et al., 1998). Nested RT-PCR confirmed the presence of an underlying *PLZF/RARα* rearrangement (see Figure 3.16). Both *PLZF-RARα* and reciprocal *RARα-PLZF* transcripts were detected as in previously described cases (Licht et al., 1995), in agreement with the concept that both fusion transcripts play a role in leukaemogenesis in this subtype of APL (Licht et al., 1995; Sitterlin et al., 1997). The sequence of *PLZF-RARα* and *RARα-PLZF* PCR products was consistent with a breakpoint within the second intron of *RARα* as occurs in t(15;17) associated APL, and a *PLZF* breakpoint within the intron separating the exons coding for the second and third zinc fingers (see Figure 3.16), as identified in 5 of 6 previously characterised cases (Chen et al., 1993a,b; Licht et al., 1995).

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Figure 3.16

Detection of PLZF-RARα and RARα-PLZF transcripts by nested RT-PCR in t(11;17)(q23;q12-21) associated APL (Case 7, Table 3.2).

PLZF-RARα and RARα-PLZF cDNA sequences are shown on the right; the positions of RARα and PLZF fusion junctions are delineated by vertical arrows.
3.6. Structure of PML nuclear bodies in APL

PML is predominantly localised to the nucleus within multiprotein structures known as PML nuclear bodies (see section 1.4; Figure 1.3). Over 10 constituents of the nuclear body have been described to date (Hodges et al., 1998), including PIC1 (UBL1, GMP1, SUMO1), a ubiquitin-homology domain protein (Boddy et al., 1996), which has been found to interact with PML (Boddy et al., 1996; Kamitani et al., 1998; Müller et al., 1998). Cells from a wide range of tissues and blasts from leukaemic subtypes other than APL which lack the t(15;17) typically demonstrate 10-30 discrete nuclear bodies when stained with PML antisera. Whereas, in APL cases associated with the t(15;17), a microparticulate pattern of PML staining is characteristic (Daniel et al., 1993; Weis et al., 1994; Dyck et al., 1994), forming the basis of a rapid diagnostic test for APL using cytospin preparations (Dyck et al., 1995). Disruption of nuclear bodies, due to an interaction between PML and PML-RARα, may promote leukaemogenesis by delocalising the putative growth suppressor PML (Mu et al., 1994) together with other nuclear body components and has been proposed to contribute to the block in myeloid differentiation that characterises the disease (Dyck et al., 1994). Treatment of such cases with ATRA leads to terminal differentiation of the leukaemic clone accompanied by normalisation of nuclear architecture (Weis et al., 1994; Dyck et al., 1994). However, rare cases of PML-RARα mediated APL have been identified that fail to respond to retinoids associated with persistence of the microparticulate PML nuclear staining pattern. On the basis of these findings it was suggested that disruption of PML nuclear bodies is critical to the pathogenesis of APL and that reconstitution of normal nuclear architecture is essential to permit differentiation in the presence of retinoids (Dyck et al., 1994). The following section considers the adaptation of the PML-immunofluorescence technique to the analysis of buffy coat smears derived from patients entered into the MRC ATRA trial as a means of identifying those with an underlying PML-RARα fusion. Furthermore, availability of buffy coat smears from a case of t(11;17) associated APL afforded the opportunity to address the role of PML nuclear bodies in the pathogenesis of APL.

3.6.1 Evaluation of PML-immunofluorescence in the MRC ATRA trial

Immunofluorescence using PML polyclonal antisera was performed on buffy coat preparations of diagnostic material derived from a series of patients entered into the MRC ATRA trial. These included 11 cases with documented t(15;17) serving as positive
controls, 15 cases with molecular evidence of cryptic PML/RARα rearrangements as determined by RT-PCR and/or FISH and 9 cases from the trial which lacked RT-PCR evidence for an underlying PML/RARα rearrangement. In addition cell lines (HL60, U937) and a series of 9 AML samples of subtypes other than M3 obtained from other MRC trials served as negative controls. PML-immunofluorescence studies of bone marrow or peripheral blood slides demonstrated the characteristic microparticulate staining pattern indicative of the presence of the PML-RARα fusion protein in 11/11 patients with the t(15;17) and 11/15 patients with cryptic PML/RARα rearrangements (e.g. Figure 3.17a). False negative results were accounted for by the absence of leukaemic cells in the peripheral blood of 3 cases, whilst severe cellular disruption revealed by phase contrast microscopy precluded meaningful analysis of the other sample. This underlines the importance of restricting analysis to diagnostic bone marrows and peripheral blood films containing leukaemic blasts, reported in conjunction with phase contrast to achieve reliable results with this methodology. In studies of non-APL cell lines and patient controls, typically less than 20 nuclear dots per cell were observed (e.g. Figure 3.17b). Similar wild-type patterns were observed in blasts from each of the remaining 9 trial patients, thereby confirming the absence of a PML/RARα rearrangement determined by cytogenetic and molecular studies and in agreement with subsequent morphological review which excluded the original clinical diagnosis of APL in these patients. Results of PML-immunofluorescence assays were routinely obtained within 4 hours, thereby confirming its suitability as a rapid diagnostic test for APL.

3.6.2 PML and PIC1 localisation in APL cases lacking the t(15;17)

The identification of the APL case with t(11;17) associated with a PLZF/RARα rearrangement (case 7, Table 3.2) afforded the opportunity to determine whether disruption of PML nuclear bodies is indeed critical to the pathogenesis of all forms of APL. This is of particular interest with respect to t(11;17)(q23;q21) associated APL, given that PLZF in common with PML has been shown to possess growth suppressor function (Pandolfi, 1996; Shaknovich et al., 1998). Furthermore, recent studies have suggested that PML and PLZF may interact in vivo (Koken et al., 1997) raising the possibilities that their growth suppressor functions might be linked and that disruption of PML nuclear bodies could provide a common pathway to the pathogenesis of APL. However, PML-immunofluorescence analysis using the polyclonal antiserum revealed a wild-type staining pattern in blasts from the t(11;17) case, suggesting that PML nuclear bodies were not disrupted in this form of APL (Figure 3.17c). This was investigated
Figure 3.17 PML immunofluorescence in AML using polyclonal antisera.

Phase contrast is shown in the left-hand panels and corresponding PML immunofluorescence on the right. In APL cases with cryptic formation of the PML-RARα fusion gene, a microparticulate pattern of PML nuclear staining was observed as shown in (a). In non-APL cases, a wild-type pattern of PML nuclear staining was detected, as shown in AML M2 blasts in (b); similar nuclear staining was observed in t(11;17) associated APL (case 7; Table 3.2), shown in (c).
Figure 3.18 PML and PIC1 localisation in AML

Dual immunofluorescence using PML monoclonal (left-hand panels) and PIC1 polyclonal (centre panels) antibodies. Images are fused in the right-hand panels; yellow signal denotes regions of PML/PIC1 colocalisation. (a) In APL cases associated with cryptic formation of the *PML-RARα* fusion gene, a microparticulate pattern of PML nuclear staining was associated with partial colocalisation with PIC1. In non-APL cases, e.g. AML M4, shown in (b) and in t(11;17)-associated APL (case 7; Table 3.2), shown in (c), PIC1 was localised to the nuclear membrane and colocalised with PML within discrete nuclear bodies.
further by dual immunofluorescence staining, using the PG-M3 PML monoclonal antibody (Flenghi et al., 1995) in combination with a polyclonal antibody for the nuclear body constituent PIC1 (UBL1). In 3 non-APL AML control cases, PML and PIC1 were colocalised within discrete nuclear bodies as shown in Figure 3.18b; as distinct from the pattern detected in a patient with a cryptic $PML/RAR\alpha$ fusion (case 5, Table 3.2) in which microparticulate PML staining was observed, with only partial colocalisation with PIC1 (Figure 3.18a). In the patient with t(11;17) associated APL, PML and PIC1 were colocalised within discrete nuclear bodies (Figure 3.18c), as observed in non-APL cases. This result has since been confirmed in vitro, whereby expression of $PLZF-RAR\alpha$ in U937 cells did not délocalise PML from nuclear body structures (Ruthardt et al., 1998).

In the present study, in addition to localisation to nuclear bodies, a perinuclear pattern of PIC1 staining was observed (see Figure 3.18b,c), consistent with its reported interaction with RanGap1 targeting it to the nuclear pore complex (Matunis et al., 1996; Mahajan et al., 1997).

Whilst it remains a possibility that disruption of nuclear bodies and delocalisation of their components may contribute to the pathogenesis of $PML-RAR\alpha$ associated APL, it is clear that delocalisation of PML from nuclear bodies is not a prerequisite for the development of all forms of this disease. Indeed, this has also been confirmed by the presence of a normal PML localisation pattern in APL cases associated with the rarer $NuMA/RAR\alpha$ (Wells et al., 1997) and $NPM/RAR\alpha$ rearrangements (Redner et al., 1997). As to whether PLZF, which is delocalised in $PML-RAR\alpha$ associated APL, is implicated in the pathogenesis of these extremely rare forms of APL remains to be determined.

3.7. Identification of $PML/RAR\alpha$ rearrangements in cases of AML lacking APL morphology

The t(15;17)(q22;q21) abnormality has been considered the diagnostic hallmark of APL and hence restricted to AML FAB type M3. However, amongst 224 AML patients treated at University College and Middlesex Hospitals between 1992 and 1998 who were considered to have FAB types other than M3, 2 cases (classified as M1 and M2) were found to have the t(15;17). RT-PCR analysis confirmed the presence of an underlying $PML/RAR\alpha$ rearrangement, thereby affording the opportunity to determine whether disruption of PML nuclear bodies and ATRA sensitivity are restricted solely to cases with typical APL morphology.
3.7.1 Clinical and morphological features

Case 1
A 36 year old male presented with a chest wall mass, initially diagnosed as non-B non-T cell high grade non Hodgkin's lymphoma, which failed to respond to 6 courses of CHOP chemotherapy. Central review of the original histology showed the malignant cells to be lysozyme positive and the diagnosis was revised to granulocytic sarcoma. Despite subsequent treatment with idarubicin and cytosine arabinoside followed by FLAG chemotherapy, bone marrow trephine revealed persistent disease. He was therefore treated palliatively with localised radiotherapy and oral chemotherapy (chlorambucil, etoposide). Shortly thereafter circulating blasts were seen and bone marrow aspirate revealed replacement by blasts with sufficient maturing cells to be classified as AML of FAB type M2. Occasional more granular cells were seen (<1%) as shown in Figure 3.19A(i) in addition to rare cells with bilobed nuclei (4%). Trephine biopsy performed at this stage demonstrated effacement of normal marrow architecture by myeloblasts, identical to those identified in the original biopsy material. In contrast to the aspirate, bilobed nuclei reminiscent of the appearances of APL were a more prominent feature (see Figure 3.19A(ii). Cytogenetic analysis in the final leukaemic phase showed 46,XY,t(15;17) in 20/20 cells with 3 metaphases showing additional abnormalities. The patient died 2 days later from a presumptive intracerebral haemorrhage.

Case 2
A 15 year old female presented with AML which appeared to be of FAB type M1, as shown in Figure 3.19B(i). No hypergranular blasts or Auer rods were present; furthermore no intense staining with myeloperoxidase was seen, see Figure 3.19B(ii). The blasts were not typical of those observed in hypogranular variant APL, exhibiting a high nuclear:cytoplasmic ratio. However, some features associated with APL were seen; cytoplasmic blebbing was noted in 54% of blasts and in a minority of cells the nuclei appeared folded, although without the classic "cottage loaf" pattern typically associated with M3variant. No differences were observed between the morphological appearances of blasts present in the peripheral blood or bone marrow aspirate. In agreement with Case 1, the bone marrow trephine showed infiltration by a population of abnormal cells with more obvious folded nuclei, see Figure 3.19B(iii). The apparent increase in lobulation of blast nuclei in the trephine specimen may reflect the sectioning technique which results in more obvious separation of the nuclear lobes displaying a pattern reminiscent of that associated with APL. Cytogenetic analysis at diagnosis showed the karyotype 47,XX,+8,t(15;17) in 13/13 cells examined. She received 4 courses of
Figure 3.19 Bone marrow aspirate and trephine appearances of AML cases with the t(15;17) classified as M1/M2.

A.
(i) Typical blasts detected in the marrow aspirate from Case 1 (section 3.7.1), classified as AML M2; a small population of granular blasts (<1%) was observed, an example of which is shown in this panel.
(ii) Bone marrow trephine revealed infiltration by blasts, some of which demonstrated nuclear lobulation.

B.
(i) Typical blasts detected in the marrow aspirate from Case 2 (section 3.7.1), classified as AML M1. The majority of blasts lacked the classic bilobed nuclei associated with M3 variant, although some evidence of nuclear folding was apparent.
(ii) Myeloperoxidase staining was less intense than typically associated with the hypogranular variant of APL.
(iii) In accordance with Case 1, bone marrow trephine revealed infiltration by blasts with more obvious nuclear lobulation.
Chapter three: Molecular characterisation of cases of suspected APL

chemotherapy in accordance with the MRC AML 12 protocol without ATRA. She relapsed 2 months after completion of therapy and was reinduced with ATRA 45mg/m²/day and FLAG chemotherapy, but died 11 days later from neutropenic sepsis and renal failure.

3.7.2 Molecular characterisation

RT-PCR and PML immunofluorescence

In both patients with the t(15;17) who lacked typical features of APL, RT-PCR performed at the same time as cytogenetic analyses confirmed the presence of underlying PML/RARα rearrangements (see Figure 3.20). This contrasted with the results of RT-PCR and FISH analyses in a patient with the t(15;17) and AML M2 who was found to have no evidence for an underlying PML/RARα rearrangement (Di Bona et al., 1996). With regard to the patients identified in the present study: case 1 was found to have a bcr 2 PML breakpoint (case b8 in section 3.3.2) and RARα-PML was not expressed; whereas in case 2 both fusion transcripts were expressed with a bcr 3 breakpoint pattern. Material was also available from case 2 for analysis by PML-immunofluorescence using the polyclonal antiserum. This revealed the classic microparticulate pattern in leukaemic blasts (Figure 3.22a), thereby demonstrating that disruption of PML nuclear bodies is not necessarily restricted to patients with M3 morphology, but is merely a secondary phenomenon reflecting the presence of the PML-RARα fusion protein.

3.7.3 Determination of differentiation response to ATRA

The differentiation response to ATRA of blasts from the case of AML M1 with the t(15;17) (case 2) was determined. Differentiation assays were performed in parallel with the NB4 cell line and blasts from a case of M3 variant APL with the t(15;17) associated with a bcr 3 PML breakpoint (case 3, Figure 3.20), which were employed as positive controls. Cells were subjected to 5 days of 10⁻⁶M ATRA, which led in all cases to significant differentiation with acquisition of mature cell function as determined by the NBT test (see Table 3.3), comparable with previous studies (Huang et al., 1988; Chomienne et al., 1990). Accordingly, morphological analysis of cytopsins made from ATRA treated cultures showed evidence of differentiation compared to untreated controls for cases 2, 3 and NB4, see Figure 3.21. However, it should be noted that some
of the cells derived from case 2 still possessed nucleoli which could reflect relatively early curtailment of this experiment necessitated by the low number of patient cells available which precluded further analyses. PML-immunofluorescence studies demonstrated persistence of the classic microparticulate staining pattern in blasts derived from cases 2 and 3, as well as NB4 cells in the absence of ATRA (Figure 3.22). Treatment for 5 days with ATRA led to a significant reduction in the number of nuclear speckles, consistent with differentiation of the leukaemic clone. In case 2 and NB4 cells the appearances had not completely reverted to normal, which may again reflect the relatively short time course of this experiment. In case 3 complete normalisation of the PML nuclear staining pattern had already occurred by day 5, indistinguishable from the distribution seen in normal cells and non-APL controls (qv Figure 3.17b).
Detection of *PML/RAR*α rearrangements in non-APL cases

![Image of gel electrophoresis](image)

**Figure 3.20**

In the left-hand two lanes, amplification products from AML Cases 1 & 2 are shown. In Case 1 (AML M2) only *PML-RAR*α fusion transcripts (bcr 2 *PML* breakpoint) were detected. In Case 2 (AML M1) *PML-RAR*α and *RAR*α-*PML* were both expressed (bcr 3 *PML* breakpoint). Also shown are RT-PCR products (bcr 1) derived from a case of M3 variant (Case 3), used as a positive control for ATRA differentiation assays (see section 3.7.3). Examples of AML cases lacking *PML/RAR*α rearrangements are shown in adjacent lanes. Positive and negative controls for the *PML/RAR*α rearrangement were provided by NB4 and HL60 cell lines, respectively; water controls for the RT and PCR steps were also included. Amplification of *RAR*α transcripts as a control for RNA integrity is shown in the lower panel.
Figure 3.21 Differentiation response to 5 days 10⁻⁶M ATRA as determined by morphological appearances.

In the right-hand panels the appearance of ATRA treated cells is shown, with untreated controls shown on the left for comparison. Leukaemic cells derived from Case 2 (AML M1 with the t(15;17)) are shown in panels a and b; cells from Case 3 (M3 variant case, see section 3.7.3) are shown in panels c and d, whilst the APL cell line NB4 is shown in panels e and f. In each case, culture in the presence of ATRA was associated with morphological changes consistent with differentiation. It should be noted that culture even in the absence of ATRA had a significant effect on the morphology of blasts derived from both Cases 2 and 3, associated with a marked increase in granularity. The majority of blasts observed in the original marrow aspirate from Case 3 were consistent with M3 variant morphology, demonstrating bilobed nuclei and cytoplasm with a faint dusting of granules (not shown), whilst appearances of blasts following 5 days culture seen in panel c are more reminiscent of hypergranular M3. Similarly, blasts derived from Case 2 became more granular in culture as shown in panel a (compare Figure 3.19 B panel i) and are more reminiscent of the hypogranular variant of APL.
Figure 3.22 PML immunofluorescence patterns in the absence or presence of ATRA

In the right-hand panels the appearance of ATRA treated cells is shown, with untreated controls shown on the left for comparison. Leukaemic blasts from a case of AML M1 with the t(15;17) (Case 2) were found to exhibit the classic microparticulate PML nuclear staining pattern (panel a), as was also observed in the M3 variant case (Case 3, shown in panel c) and the NB4 APL cell line (panel e). In each case the differentiation response induced by 5 days treatment with ATRA was associated with a reduction in the number of nuclear speckles (shown in panels b, d and f, respectively).
**Table 3.3**: Determination of differentiation response to ATRA by NBT assay.

Cultured cells were incubated with and without 500nM 12-O-tetradecanoylphorbol 13-acetate for 30mins at 37°C to stimulate NADPH oxidase activity in the presence of 0.05% (v/v) NBT. Positive cells were identified by microscopy. No positive cells were seen in unstimulated control samples. A significant differentiation response of blasts from the AML case with M1 morphology and the t(15;17) (case 2) induced by ATRA was confirmed, with results of the NBT assay matching those associated with the NB4 control. A population of NBT positive cells was detected amongst M3 variant cells (case 3) which had not been exposed to ATRA; this was most likely due to spontaneous differentiation as no cells of monocytic lineage were observed in the corresponding cytospin preparation.
CHAPTER FOUR: EVALUATING THE ROLE OF MOLECULAR MONITORING FOR RESIDUAL DISEASE BY RT-PCR IN PATIENTS ENTERED INTO THE MRC ATRA TRIAL

For many years it has been appreciated that APL constitutes a subtype of AML with relatively favourable prognosis, associated with low levels of chemo-resistance and leukaemic relapse (Keating et al., 1988; Samuels et al., 1988; Swansbury et al., 1994; Dastugue et al., 1995; Grimwade et al., 1998a). Indeed, attempts to increase survival in this group through generalised application of dose intensification using BMT in first remission proved unsuccessful, with any reduction in relapse risk being offset by procedure related mortality (Burnett et al., 1998). On this basis BMT is now typically reserved for the treatment of relapse. Over the last decade, ATRA therapy has led to a dramatic improvement in outcome for patients with APL (Fenaux et al., 1997a,b; Tallman et al., 1997). Indeed, recent studies have demonstrated that only 10-20% patients relapse following treatment with prolonged retinoid therapy in conjunction with combination chemotherapy (Mandelli et al., 1997, Burnett et al., 1997). In order to achieve further improvements in cure rates in APL, it has become increasingly important to identify the relatively small subgroup of patients at particular risk of relapse who at present cannot be successfully identified by pretreatment characteristics and who could benefit from more intensive treatment in first remission.

The subsequent chapter evaluates the role of nested RT-PCR as a means of detecting minimal residual disease (MRD) and predicting relapse risk in a large group of patients with newly diagnosed APL treated in the MRC ATRA trial. In view of the greater sensitivity afforded by the \textit{RARa-PML} assay, this was performed in parallel with the more conventional assay for \textit{PML-RARa} to determine whether residual disease detection was enhanced. In addition, molecular monitoring was performed in a series of patients with relapsed APL who achieved a second remission to determine whether this approach can also serve to identify those individuals most likely to benefit from subsequent bone marrow transplantation procedures.
Chapter four: MRD detection in APL

4.1. MRD detection using nested RT-PCR assays for \textit{PML-RAR}\alpha and \textit{RAR}\alpha-\textit{PML} fusion transcripts, following ATRA and chemotherapy treatment

4.1.1 Patient characteristics

Molecular monitoring studies using nested RT-PCR were performed in a series of 105 ATRA trial patients to determine the kinetics of achievement of molecular remission following treatment and its relationship to the risk of subsequent relapse. The analysis was restricted to patients with APL with confirmed \textit{PML/RAR}\alpha rearrangements, who attained morphological CR and received at least 2 courses of consolidation therapy as outlined in Figures 2.3-2.5 (AML 10 n=58, AML 12 n=37, AML 11, n=10). In 99/105 patients, availability of suitable material permitted determination of \textit{PML} breakpoint and \textit{RAR}\alpha-\textit{PML} expression pattern (61, 3’ breakpoint: 43 \textit{RAR}\alpha-\textit{PML} +ve, 18 -ve; 38, 5’ breakpoint: 29 \textit{RAR}\alpha-\textit{PML} +ve, 9 -ve); in the remainder the diagnosis was confirmed cytogenetically by the presence of the t(15;17). Whilst peripheral blood was found to be satisfactory for RT-PCR analyses to establish the presence of \textit{PML-RAR}\alpha rearrangements for diagnostic purposes, only bone marrow samples with a minimum of 1 x 10^7 cells were considered suitable for MRD assessment. Material was requested following haemopoietic recovery after each course of chemotherapy, with a median of 2 analyses per patient (range 1-4). There was no significant difference in outcome between patients in whom molecular monitoring was performed and the group of 58 confirmed APL cases achieving CR and completing therapy from whom no material was received for molecular analysis (overall survival at 3yrs 87% vs 83%). Also, no significant difference was observed in the rate of disappearance of \textit{PML-RAR}\alpha and/or \textit{RAR}\alpha-\textit{PML} fusion transcripts between patients randomised to Extended or Short ATRA (data not shown).

4.1.2 Parallel assessment of \textit{PML-RAR}\alpha and \textit{RAR}\alpha-\textit{PML} assays for MRD detection

Detection rates of \textit{PML-RAR}\alpha and \textit{RAR}\alpha-\textit{PML} fusion transcripts following each course of chemotherapy are shown in Figure 4.1. In patients expressing \textit{RAR}\alpha-\textit{PML}, disappearance of these reciprocal derived transcripts was frequently found to lag behind \textit{PML-RAR}\alpha, consistent with the increased sensitivity associated with the \textit{RAR}\alpha-\textit{PML} assay for both \textit{PML} breakpoint patterns, noted in dilution studies of the NB4 cell line and diagnostic patient material (see section 3.1.2). Employment of the \textit{RAR}\alpha-\textit{PML} assay
Rate of disappearance of $PML\text{-}RAR\alpha$ and $RAR\alpha\text{-}PML$ fusion transcripts in MRC ATRA trial patients attaining complete remission.

The upper figure considers the results of molecular monitoring using both assays in 105 patients, irrespective of whether $RAR\alpha\text{-}PML$ was expressed at the time of diagnosis. In the lower figure, results are displayed for the group of patients ($n=72$) in whom reciprocal $RAR\alpha\text{-}PML$ transcripts were known to be expressed. Numbers of patients assessed at each timepoint are displayed above columns.
Combined use of $PML-RAR\alpha$ and $RAR\alpha-PML$ assays enhances detection of minimal residual disease

Figure 4.2: Amongst patients in whom $RAR\alpha-PML$ was expressed, disappearance of these reciprocal derived transcripts was frequently found to lag behind $PML-RAR\alpha$, consistent with the increased sensitivity associated with the $RAR\alpha-PML$ assay for both $PML$ breakpoint patterns, noted in dilution studies of the NB4 cell line and diagnostic patient material (see Figures 3.3 & 3.4). For example, in APL patient 1, $PML-RAR\alpha$ and $RAR\alpha-PML$ fusion transcripts with a 3' $PML$ breakpoint pattern were detected at diagnosis. Subsequent bone marrow examinations performed following second, third and fourth courses of chemotherapy revealed solely $RAR\alpha-PML$ transcripts. The patient received no further therapy and a bone marrow aspirate performed 2 years later revealed no evidence of residual disease. In APL patient 2, $PML-RAR\alpha$ and $RAR\alpha-PML$ fusion transcripts with a 5' $PML$ breakpoint pattern were detected at diagnosis. A 474bp $RAR\alpha-PML$ transcript was still detectable following the second course of chemotherapy, this was confirmed by sequence analysis to represent fusion of $RAR\alpha$ exon 2 to $PML$ exon 4. No evidence of residual disease was detectable following third or fourth courses of chemotherapy. Also shown are analyses of 1/1000 dilution of NB4 as a positive control, HL60 negative control, with water controls to exclude contamination at RT and PCR steps. RT-PCR to detect $PML$ transcripts was performed in parallel to confirm RNA integrity.
in addition to the more conventional method for \textit{PML-RAR}\alpha\ led to the detection of disease-related transcripts in an additional 20\% (21/105) of patients whilst in morphologic CR, and included 4 patients with 5' \textit{PML} breakpoints as well as 17 with 3' breakpoints (see Figure, 4.2).

4.1.3 Predictive value of molecular monitoring at the end of consolidation therapy

Amongst the 35 ATRA trial patients assessed for the presence of MRD following the 4th course of chemotherapy, none were found to have detectable \textit{PML-RAR}\alpha\ fusion transcripts (see Figure 4.1, upper panel); nevertheless 11/35 ultimately relapsed. It was then considered whether the more sensitive \textit{RAR}\alpha-\textit{PML} assay could have predicted the subsequent relapses. This assay had been informative at diagnosis in 8 of the relapsing patients (\textit{PML} breakpoint: 3' \textit{n}=3, 5' \textit{n}=5), however \textit{RAR}\alpha-\textit{PML} transcripts were detected in only one of them (5' \textit{PML} breakpoint) following completion of therapy.

Overall, 22 of the 35 patients assessed at the end of treatment were known to have had an informative reciprocal \textit{RAR}\alpha-\textit{PML} assay at diagnosis (see Figure 4.1, lower panel). \textit{RAR}\alpha-\textit{PML} transcripts were detected in 2 of these patients immediately following completion of therapy: 1 patient with a 3' \textit{PML} breakpoint subsequently tested PCR negative in a further marrow sample received 24 months later with no intervening treatment (see Figure 4.2) and remains in remission 3.5 years after completion of therapy, whilst the other patient (mentioned above) relapsed within 7 months. Twelve patients found to express \textit{RAR}\alpha-\textit{PML} at diagnosis (3' \textit{PML} breakpoint, \textit{n}=9, 5', \textit{n}=3) and maintaining long-term remission were also evaluated for both fusion transcripts in the first year following completion of therapy, none were found to have detectable fusion transcripts.

4.1.4 Predictive value of molecular monitoring during consolidation therapy

RT-PCR profiles derived from molecular monitoring studies performed following induction and during consolidation therapy were compared between 26 ATRA trial patients who ultimately relapsed and 79 remaining in complete remission (median follow-up 44 months, range 4.8-65). The detection rate of disease related transcripts following each course of chemotherapy was greater amongst the group who ultimately relapsed, see Figure 4.3. Amongst the whole group, detection of transcripts at any stage...
Figure 4.3

Rate of disappearance of disease related transcripts (*PML-RARα* &/*RARα-PML*) in ATRA trial patients maintaining continuous complete remission (CR) or ultimately relapsing.

Numbers displayed above the columns refer to patients in each group assessed at a particular timepoint.
<table>
<thead>
<tr>
<th>Time of PCR assessment</th>
<th>Post chemo. course 1</th>
<th>Post chemo. course 2</th>
<th>Post chemo. course 3</th>
<th>Post chemo. course 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR status:</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>(detection of PML-RARα &amp;/or RARα-PML)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of patients</td>
<td>11</td>
<td>42</td>
<td>34</td>
<td>18</td>
</tr>
<tr>
<td>Relapse risk (% at 3 years)</td>
<td>18%</td>
<td>25%</td>
<td>18%</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td>25%</td>
<td>25%</td>
<td>57%**</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td>50%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1

Predictive value of molecular monitoring following induction and consolidation chemotherapy in a group of 105 MRC ATRA trial patients. Detection of disease-related transcripts (PML-RARα and/or RARα-PML) immediately after the third course of chemotherapy was associated with a significant increase in relapse risk, compared to the group with no evidence of MRD at this stage. ** p<0.01
Figure 4.4 Relapse risk by presence of minimal residual disease post course 3

At risk:

<table>
<thead>
<tr>
<th></th>
<th>P-R and/or R-P+</th>
<th>P-R and R-P-</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. Patients</td>
<td>7</td>
<td>69</td>
</tr>
<tr>
<td>Obs.</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Exp.</td>
<td>1.1</td>
<td>19.9</td>
</tr>
</tbody>
</table>

p=0.004

No. Events

- % relapsing
- 57%
- 25%

Years from remission

- At risk:
  - P-R and/or R-P+: 7
  - P-R and R-P-: 69

- 0
- 1
- 2
- 3
Figure 4.5 Survival by presence of minimal residual disease post course 3

<table>
<thead>
<tr>
<th></th>
<th>No. Patients</th>
<th>No. Events</th>
<th>Obs.</th>
<th>Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-R and/or R-P +</td>
<td>7</td>
<td>3</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>P-R and R-P -</td>
<td>69</td>
<td>10</td>
<td>12.1</td>
<td></td>
</tr>
</tbody>
</table>

p=0.02

At risk:

P-R and/or R-P +  | 7
P-R and R-P -    | 69

Years from entry

% still alive

- P-R and/or R-P +
- P-R and R-P -

- 89%
- 57%
- 57%
Chapter four: MRD detection in APL

following induction or during consolidation therapy was associated with an increased risk of relapse, see Table 4.1. This trend was found to be most predictively useful following the third course of chemotherapy when most patients were evaluable, coinciding with the timing of bone marrow harvesting in the AML 10 and 12 trials. Detection of disease-related transcripts at this stage predicted a significantly increased risk of relapse and poorer overall survival, see Figures 4.4, 4.5. Furthermore, each of the patients with evidence of residual disease at this time had low presenting leucocyte counts, confirming delayed clearance of disease-related transcripts as an independent prognostic factor in APL.

These findings suggest that the kinetics of achievement of molecular remission have an important bearing on the risk of subsequent relapse. This is in agreement with data correlating post induction blast count with prognosis in AML (Burnett et al., 1998) and also with residual disease studies performed in ALL using PCR and both diseases using flow cytometry (Brisco et al., 1994; San Miguel et al., 1998; Coustan-Smith et al., 1998; Cave et al., 1998). However, the benefit of the present study is the demonstration of this phenomenon in a homogeneous disease entity, independent of recognised pretreatment prognostic factors. Furthermore, RT-PCR monitoring in APL affords the added advantage of providing an objective confirmation of morphologic remission, aiding distinction of marrow regeneration from persistent disease which can be particularly problematic in this subtype of AML (Stone & Mayer, 1990).

4.1.5 RT-PCR analysis performed at relapse

Amongst 12 patients from whom bone marrow was available from the time of relapse, no discrepancy was observed in either PML breakpoint or RARα-PML expression pattern in comparison to the time of initial presentation. This included one patient in whom relapse also involved the CNS, confirmed by the identification of PML-RARα fusion transcripts within cells derived from the CSF (see Figure 4.6). The identification of such patients has raised concerns that ATRA may increase the risk of subsequent relapse at extramedullary sites through modulation of intercellular adhesion molecules (Weiss & Warrell, 1994; Evans et al., 1997); indeed analysis of UK MRC and Italian AIDA studies has revealed that 10% relapses in APL cases treated with ATRA and chemotherapy involve extramedullary sites (Evans & Grimwade, 1998).
Central nervous system relapse in an APL patient with the PML/RARα rearrangement, previously treated with ATRA

Figure 4.6

Molecular monitoring for PML-RARα fusion transcripts was performed throughout the disease course of an MRC ATRA trial patient who ultimately relapsed in the CNS. RT-PCR and PML-RARα junctional oligoprobe analysis of diagnostic bone marrow were consistent with a bcr 1 PML breakpoint. Disease-related transcripts were also detected in the bone marrow following the third course of chemotherapy, and the patient relapsed with CNS symptoms 2 months after completion of consolidation therapy. CSF cytospin at this time demonstrated numerous promyelocytic blasts. RT-PCR of total RNA extracted from CSF cells revealed expression of PML-RARα fusion transcripts with an identical PML breakpoint pattern to that identified at diagnosis. Bone marrow RT-PCR was also positive at this stage, consistent with detection of 15% abnormal promyelocytes on morphologic examination. Also shown are RT-PCR analyses for NB4 and HL60 cell line controls, together with water controls to exclude contamination at the reverse transcription and PCR steps.
4.2 Molecular monitoring in APL patients undergoing bone marrow transplantation

In addition, molecular monitoring was evaluated in a series of 9 patients with relapsed APL, who achieved second remission with combinations of ATRA and chemotherapy and proceeded to bone marrow transplantation (BMT) (see Figure 4.7). Eight patients (A1-8) received an autologous bone marrow transplant (ABMT), using marrow harvested in first remission taken prior to the fourth course of chemotherapy, whilst in patient B1 an allogeneic BMT was performed using a matched sibling donor. Seven patients were derived from the MRC ATRA trial (A1-6, B1), whilst the remaining patients (A7,A8) initially presented before the start of the latter trial and received ATRA and AML 10 chemotherapy as first line therapy.

In 7 patients molecular monitoring was performed immediately before BMT. Four patients proceeded to ABMT with no disease-related transcripts detectable by RT-PCR (A1,2,4,6); in each case RT-PCR undertaken on an aliquot of the harvested marrow was also negative and these patients remain in remission 7-48 months post transplant. Three patients were found to have evidence of residual disease immediately prior to transplant (A3,5,B1). In 2 of these patients (A3,B1), further analyses performed 1 month post BMT revealed no disease-related transcripts, consistent with successful cytoreduction by the conditioning regimen and both patients remain in remission for 29 and 47 months, respectively. The other patient with evidence of residual disease prior to ABMT (A5), received a PCR negative graft, but relapsed and died within 3 months of transplant. In 2 patients no material was available to determine PCR status immediately prior to ABMT (A7,8). However, both were found to have evidence of residual disease as determined by FISH analyses performed 2.5-4.5 months post ABMT. In both patients interphase FISH (performed by H.Kempski, Great Ormond Street Hospital) was undertaken using a chromosome 17 paint (Oncor) and/or locus specific probes for the PML-RARα fusion (Vysis); percentages of cells with a pattern indicative of the presence of the t(15;17) are shown on Figure 4.7, these compare to < 2% positive cells detected in normal and non-APL control marrows. Whilst it was not possible to determine the relative contributions of graft contamination or incomplete elimination of the disease by the conditioning regimen to account for the presence of residual disease post ABMT, in the light of previous work showing that re-emergence or persistence of PML-RARα fusion transcripts detectable by the more sensitive RT-PCR technique heralds relapse (Lo Coco et al., 1992), relapse in these patients was considered inevitable and they were recommenced on ATRA accordingly. Indeed, the likelihood of subsequent clinical
Molecular monitoring in patients undergoing bone marrow transplantation in second CR
Patients A1-8 received autologous bone marrow transplants, whereas patient B1 received marrow from a matched sibling donor (allo BMT). *PML* breakpoint patterns are indicated in patients in whom RT-PCR was performed at the time of relapse. Numbers adjacent to FISH monitoring results refer to the percentage of residual APL cells.
Abbreviations: CR, complete remission; CCR, continuous CR from time of bone marrow transplant.
relapse in these patients in the absence of further therapy has been supported by more recent studies which demonstrated that detection of \textit{PML-RAR\alpha} fusion transcripts by RT-PCR 3 months post ABMT was predictive of relapse (Meloni \textit{et al.}, 1997; Román \textit{et al.}, 1997). In patients A7 and A8, subsequent analysis of bone marrow aspirates by RT-PCR after prolonged ATRA therapy revealed no evidence of disease-related transcripts presumably reflecting differentiation and subsequent apoptosis of residual leukaemic cells. Indeed both patients remain in continuous CR at least 2 years following cessation of retinoid therapy.

\textbf{4.3 Implications of results of molecular monitoring studies in APL}

The results of molecular monitoring studies described in section 4.1 demonstrate that the conclusions drawn from early molecular monitoring studies (see section 1.8.2) which included significant numbers of patients treated in relapse or with ATRA as single agent therapy (Lo Coco \textit{et al.}, 1992; Miller \textit{et al.}, 1992) cannot effectively be extended to patients treated with ATRA and chemotherapy. Indeed, it is now clear from this study and others (Miller \textit{et al.}, 1993; Fukutani \textit{et al.}, 1995; Mandelli \textit{et al.}, 1997) using assays of comparable sensitivity to those employed in the original studies, that the majority of patients treated with ATRA and chemotherapy ultimately become PCR negative. Furthermore, it is clear from this study and also more recent data from the Italian group (Diverio \textit{et al.}, 1998), that the majority of patients treated in this way, who ultimately relapse have no evidence of \textit{PML-RAR\alpha} transcripts following completion of therapy. The relative insensitivity of the \textit{PML-RAR\alpha} assay has been noted previously and was attributed largely to inefficiency at the reverse transcription stage (Seale \textit{et al.}, 1996). Sensitivity was found to be significantly improved by the incorporation of an RNA denaturation step and by prolonging the incubation time for the RT step (Seale \textit{et al.}, 1996); measures which were both incorporated in the present study. In view of the greater sensitivity of the \textit{RAR\alpha-PML} assay, it was of interest to determine whether parallel use of this assay could identify the subgroup at risk of relapse. However, this approach was also unsuccessful in the majority of cases tested following completion of therapy. Whilst it is possible that some false negative results were obtained due to poor sample quality (despite satisfactory \textit{PML} and \textit{RAR\alpha} controls), the results presented here suggest that the majority of patients ultimately relapsing after ATRA and combination chemotherapy as employed in the MRC trial have levels of residual disease below the level of sensitivity of \textit{PML-RAR\alpha} and \textit{RAR\alpha-PML} assays (i.e. < 1 APL cell in $10^5$ marrow cells) following completion of therapy.
Interestingly, attempts to increase the sensitivity of \textit{PML-RAR\textalpha} and \textit{RAR\textalpha-PML} assays still further by increasing the efficiency of the RT step combined with a "hot start" PCR has led to the detection of disease related transcripts in patients in long term remission (Tobal \textit{et al.}, 1995; Tobal & Yin, 1998). These results are in accordance with previous studies which have detected \textit{AML1-ETO} transcripts in patients with t(8;21) associated AML in long term remission (Nucifora \textit{et al.}, 1993). This would imply that a number of patients considered to be cured of leukaemia retain a population of cells harbouring the disease associated transcript. Such cells could be prevented from further proliferation by immunological mechanisms. Alternatively, it is possible that transcripts such as \textit{AML1-ETO} or \textit{PML-RAR\textalpha/RAR\textalpha-PML} are insufficient to mediate leukaemogenesis in their own right and leukaemic remission reflects the eradication of clones of cells which acquired additional oncogenic events. It would appear that inter-study variation in the detection of disease related transcripts in patients in long term remission is more likely to reflect differences in the sensitivity limits of the respective assays rather than necessarily implying differences in tumour biology associated with remission. Whilst improvement in the sensitivity of nested RT-PCR assays is of interest as a means to investigate the mechanisms underlying remission, thus far it has failed to yield clinically relevant information, hampering the distinction of patients remaining clinically disease free from those who relapse. This may be addressed by adopting a semi-quantitative nested RT-PCR approach; however, this is not best suited for analysis of material from multicentre clinical trials using conventional methodology, but may be feasible in the future using "real time" PCR techniques (Marcucci \textit{et al.}, 1998).

The present study has shown that determination of PCR status immediately following completion of combination therapy with ATRA and chemotherapy is not prognostically useful. However, it also demonstrated that parallel assessment using \textit{PML-RAR\textalpha} and \textit{RAR\textalpha-PML} assays can enhance MRD detection and suggests that the rate of disappearance of disease related transcripts is of independent prognostic value. Amongst a group of 105 patients treated in accordance with the MRC ATRA trial, delayed clearance of disease-related transcripts was associated with an increased risk of relapse, associated with a poorer prognosis. Whilst this result should be confirmed in a larger group of uniformly treated patients including extended ATRA therapy, it would suggest that molecular monitoring can identify subgroups of APL at high risk of relapse. It is possible that this group of patients could benefit from additional consolidation therapy, including BMT in first remission. As to whether such an approach is superior to a strategy involving molecular monitoring post consolidation associated with reinstitution.
of treatment at the point of molecular relapse, which has been shown to herald clinical relapse (Diverio et al., 1998), remains to be determined.

There is relatively little available data relating to molecular monitoring performed in patients with APL undergoing BMT, reflecting the relative infrequency of relapses in this condition. The favourable outcome identified in 4 patients with no detectable disease-related transcripts prior to transplant, is in accordance with data from the Italian group in which 7/8 such patients remain in remission from APL following ABMT (Meloni et al., 1997). The outcome of patients with molecular evidence of residual disease prior to transplant is less certain and may in part be influenced by the degree of contamination in the graft. In the Italian study 7 patients with residual disease prior to ABMT, received marrow with RT-PCR evidence of disease contamination, all of whom subsequently relapsed. Our study, indicates that long term relapse free survival is feasible in patients with evidence of residual disease prior to transplant, in the context of allogeneic BMT or ABMT using graft material lacking detectable fusion transcripts by RT-PCR. In addition, previous studies support a role for molecular monitoring following BMT as a means of determining outcome. In these studies patients in long term remission after BMT had no detectable PML-RARα fusion transcripts by RT-PCR, whilst detection of residual disease 3 months post BMT predicted relapse (Meloni et al., 1997; Román et al., 1997). This would suggest that the evidence of residual disease detected by FISH, 4 months post ABMT in patients A7 and A8 would have been unlikely to resolve spontaneously in the course of time in the absence of ATRA therapy. The successful eradication of residual disease post transplant in these patients such that subsequent RT-PCR analyses revealed no evidence for disease related transcripts has much wider implications for the management of patients with APL, suggesting potential roles for retinoids administered after completion of first-line therapy, given either as maintenance or instituted at the point of molecular relapse. Despite combined treatment with ATRA and chemotherapy, at least 10-20% of patients with APL ultimately relapse, limiting their chance of long term cure. As ATRA has been demonstrated to successfully eliminate residual disease present at sufficient levels to be detectable by FISH one would expect that retinoids could also be efficacious as maintenance therapy post consolidation, since the present study has shown that the majority of patients relapsing following ATRA and chemotherapy have levels of disease below that detectable by conventional nested RT-PCR assays when tested immediately following completion of therapy. Indeed, the recent Intergroup study (Tallman et al., 1997) did suggest a beneficial effect for maintenance therapy with ATRA. As to whether maintenance retinoid confers an overall significant survival advantage in patients who have received
prolonged ATRA as a component of induction remains to be determined. This is particularly pertinent in the light of recent studies identifying patients previously exposed to ATRA, who are resistant to further ATRA therapy due to acquisition of mutations in the ligand-binding domain of the PML-RARα fusion protein (Imaizumi et al., 1998; Ding et al., 1998).

An alternative strategy to the indiscriminate use of retinoid maintenance therapy could also be of value; namely routine molecular monitoring by RT-PCR post consolidation with institution of ATRA (and/or chemotherapy depending on acquisition of mutations in PML-RARα) solely in patients with evidence of molecular relapse as a means of preventing subsequent clinical relapse. Studies in patients with CML post alloBMT have already demonstrated that treatment with donor leucocyte infusions at the point of molecular relapse is more efficacious than treatment instituted at the time of frank haematological relapse (Van Rhee et al., 1994). Since frank haematological relapse in APL carries a significant risk of haemorrhagic death, treatment at the point of molecular relapse could lead to further improvements in overall survival. However, due to the relative infrequency of relapses in APL, it is clear that resolution of these issues, particularly establishing the optimal timing and duration of ATRA therapy and its role in the treatment of relapse, is likely to require even larger clinical trials involving collaboration between national and international trial groups.
CHAPTER FIVE: CD2 EXPRESSION IN ACUTE PROMYELOCYTIC LEUKAEMIA

With more widespread application of immunophenotyping techniques to facilitate leukaemia diagnosis, it became apparent that antigens previously considered lymphoid-specific could be detected in cases of AML; whilst conversely, expression of myeloid associated antigens was identified in some cases of ALL. This has prompted considerable debate for over a decade, as to whether this phenomenon indicates that such leukaemias represent expansions of rare stem cells that co-express lymphoid and myeloid antigens or alternatively arise in lineage-committed cells in which the presence of "inappropriate" surface markers reflects aberrant gene expression associated with leukaemogenesis (Greaves et al., 1986; Smith et al., 1983). A number of recent immunophenotyping studies have highlighted expression of the T cell marker CD2 in a subgroup of patients with APL; this is particularly intriguing since this subtype of AML is relatively well differentiated. In order to begin to address the mechanisms underlying this phenomenon, APL samples were screened by flow cytometry using a panel of antibodies designed to detect myeloid, T and B lineage associated antigens. Furthermore, immunophenotype analysis was extended to quantitate the level of CD2 expression on APL blasts relative to expression levels at the surface of normal T lymphocytes. Finally, the regulation of CD2 expression in APL was investigated by DNase I hypersensitivity assays, comparing chromatin configuration surrounding 5' and 3' regulatory elements of the gene in a series of APL cases, with other forms of acute and chronic leukaemia as determined by analysis of primary clinical material and a variety of cell lines.

5.1. Immunophenotype analysis

5.1.1 Confirmation of surface expression of CD2 in APL

In order to confirm reports of CD2 expression in APL and to characterise cell populations used for subsequent DNase I hypersensitivity assays, immunophenotype analysis was performed in a series of APL cases, in material derived from other forms of acute and chronic leukaemias and in a variety of haemopoietic cell lines (as shown in Table 5.1). In addition to CD2, blasts were examined for expression of the stem cell
Chapter five: CD2 expression in APL

marker CD34, myeloid markers CD13, CD33 and CD117 and the B lymphoid marker CD19. Cell suspensions were double labelled using a fluorescein isothiocyanate (FITC) conjugated CD2 antibody in combination with phycoerythrin (PE) conjugated CD34, CD13, CD33, CD117, CD19 and CD3 antibodies in order to identify antigen co-expression by the cell population of interest. Using a gate applied to the leukaemic population CD2/CD13, CD2/CD33, CD2/CD34 and CD2/CD117 combinations were used to confirm or exclude CD2 expression by myeloid blasts. CD19 was included as a positive control for analysis of B lineage leukaemias and cell lines; whilst, the CD2/CD3 antibody combination was used to identify normal T lymphocytes. Measurement of the percentage of CD2+/CD3+ cells amongst the whole cell population was used to determine the degree of T lymphocyte contamination within material used for DNase I hypersensitivity assays. In view of the concern that significant T lymphocyte contamination could prevent reliable assessment of the chromatin structure surrounding CD2, samples found to have ≥5% lymphocytes were excluded from DNase I hypersensitivity analyses (discussed below).

Overall, immunophenotype analysis was performed in clinical material derived from 10 APL cases (9 variant morphology, P1-P9; 1 classical, P10), 1 case of AML M1 (P11) and from 2 cases of B lineage leukaemia (B-PLL, n=1-P12; cALL, n=1-P13). CD2 expression was detected in 6/9 cases with hypogranular variant APL (M3v); in 5 patients the majority of leukaemic blasts were found to co-express CD2, CD13 and CD33 (see Figure 5.1), whilst in the remaining case only a minority expressed CD2. In 4 cases of M3v, CD2 expression was accompanied by CD117, 3 of which also expressed the stem cell marker CD34. Furthermore, 1 of these CD34 positive cases of M3v (P2) also co-expressed CD3 and CD19. All other cases of APL were CD3 and CD19 negative. In the remaining M3v patients (Cases P6-8) and the classical M3 case, no significant expression of CD2 was detected on leukaemic blasts (see Figure 5.2). Immunophenotyping was also performed in a case of AML M1, revealing CD2 expression in a minority of blasts; whilst cases with B lineage malignancies (prolymphocytic leukaemia - PLL, common ALL - cALL) were confirmed to be CD2 negative.

Immunophenotyping was also performed in a series of haemopoietic cell lines in order to correlate surface expression of CD2 with chromatin structure surrounding the gene, as determined by subsequent DNase I hypersensitivity assays. CD2 expression was confirmed in the mature Jurkat T cell line, whilst the remaining cell lines including B lineage Daudi, and the myeloid cell lines HL60, U937, K562, HEL and the APL cell line
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NB4 were found to lack surface expression of CD2 (Table 5.1). In addition, normal donor peripheral blood T cells which had been cultured in the presence of interleukin 2 (IL-2) and phytohaemagglutinin (PHA), in order to stimulate CD2 expression, were studied. Immunophenotyping of the latter confirmed the majority of cells to be CD2+/CD3+, with minimal B cell contamination, indicated by the presence of 5% cells expressing CD19.
### Table 5.1 Immunophenotype analysis in APL and haemopoietic cell lines

For each analysis involving leukaemic samples gating was performed on the blast cell population; hence percentages refer to proportion of leukaemic cells expressing a particular antigen. N/A, not applicable; N/D, not determined.

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Figure. 5.1

CD2 expression in hypogranular variant APL, as shown by case Pl.

A: Forward scatter/ Side scatter (FSC/SSC) dot plot.
B: Isotypic controls
C: Dot plot showing co-expression of CD2/CD13
D: Dot plot showing co-expression of CD2/CD33
Figure. 5.2

Lack of CD2 expression in hypogranular variant APL, as shown by case P7.

A: Forward scatter/ Side scatter (FSC/SSC) dot plot.
B: Isotypic controls
C,D: Dot plots showing lack of CD2 co-expression in the CD13 and CD33 positive blast cell population.
Chapter five: CD2 expression in APL

5.1.2 Quantification of CD2 expression by flow cytometry

Having confirmed CD2 expression in a series of cases of the hypogranular variant of APL, it was of interest to determine the relative level of surface expression in comparison to the T cell line Jurkat or peripheral blood T lymphocytes derived from normal healthy donors in the presence or absence of IL-2/PHA stimulation (Table 5.2). Standard microbeads with different capacities to bind mouse immunoglobulins were used to convert the mean fluorescence intensity values of blast or T lymphocyte populations into the number of antigen molecules per cell, measured as antibody binding capacity (ABC). For analysis of T lymphocytes, the CD2+/CD3+ population was gated, whereas for determination of CD2 expression on leukaemic blasts, the analysis was restricted to the CD2+/CD33+ population.

CD2 surface expression on unstimulated T lymphocytes derived from normal donors \( n=4, \text{N1-N4} \) was found to be comparable to levels established by a previous study (Ginaldi et al., 1996). Similar levels of expression were documented in the 7 cases of CD2 positive AML (6 M3v cases, and 1 AML M1 case) and in the Jurkat T cell line. However, a dramatic increase in surface expression of CD2 was detected in donor T lymphocytes cultured in the presence of IL-2 and PHA.

Therefore, in summary, immunophenotype analyses presented here are in accordance with a recent large study, which documented surface expression of CD2 in 58% patients with the hypogranular variant form of APL, and correlated CD2 positivity with expression of CD34, CD19 and presence of a bcr 3 PML breakpoint pattern (Guglielmi et al., 1998). However, the quantitative analysis presented here further extends these findings, indicating that CD2 detection on APL blasts reflects the presence of comparable levels of surface antigen to that expressed on unstimulated normal T lymphocytes. Since a previous report confirmed that detection of CD2 is correlated with the relative level of gene expression, as determined by Northern analysis (Biondi et al., 1995), it was of interest to further investigate the regulation of CD2 in APL by determining the chromatin structure surrounding the gene using DNase I hypersensitivity assays.
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**Cell lines/**
**cultured peripheral T cells**

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cultured in IL-2/PHA

**AML blasts**

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**Table 5.2**
Quantitation of surface CD2 expression in APL, in relation to haemopoietic cell lines and normal T lymphocyte controls.
5.2. Determination of the chromatin structure surrounding the CD2 gene in APL

A number of previous studies have sought to determine the mechanisms underlying regulation of CD2 expression during lymphoid differentiation. One approach was the use of DNase I hypersensitivity assays to identify the important regulatory regions and to establish the chromatin configuration surrounding the gene in T and B cell lineages (Wotton et al., 1989). Three hypersensitive sites flanking the CD2 gene were identified, corresponding to key regulatory elements (see Figure 5.3). DHS 2 is situated approximately 50bp upstream from the transcription start site, corresponding to the minimal promoter. DHS 1 is situated approximately 1.8 kb further 5' related to a region that includes downstream silencer and upstream activator (or de-repressor) activities, as defined by CAT assays (Outram, 1996). Hypersensitive sites identified in the 3' flanking region (DHS 3) correspond to the enhancer and locus control elements (Wotton et al., 1989). Previous analyses of a variety of B and T lymphoid and non-haemopoietic cell lines had suggested that hypersensitivity in the 3' flanking region was restricted to the T cell lineage. DHS 3 was detected in CD2 negative KG 1 T cells and also in more mature CD2 positive Jurkat cells; whilst this site was absent in CD2 negative B cell and non-haemopoietic cell lines (Wotton et al., 1989) (see Figure 1.6). Furthermore, DHS 1 and DHS 2 sites appeared to be restricted to CD2 positive T cell lines and peripheral blood T lymphocytes (Wotton et al., 1989; Outram, 1996). In view of recent reports of CD2 expression in cases of AML, particularly the hypogranular variant form of APL, it was of interest to characterise the chromatin structure surrounding the CD2 locus in myeloid cell lines, and in blasts derived from APL patients. To our knowledge, this is the first study which has investigated regulation of chromatin structure in primary leukaemic blasts.

DNase I hypersensitivity assays were performed as previously described (Wotton et al., 1989) (see section 2.6). DNA was prepared from nuclei digested with a series of DNase I concentrations including an untreated control; digested with an appropriate restriction enzyme (Bam HI for 5' sites and Eco RI for DHS 3) and analysed by Southern analysis to identify hypersensitive sites. Details of the CD2 cDNA probes used are presented in Figure 5.3. Probe A was used to detect 5' hypersensitive sites; this probe detects a 9kb genomic fragment containing the first and second exons. The detection of additional bands of 2.6 and 0.8kb in digests of DNA prepared from DNase I treated nuclei was indicative of the presence of DHS 1 and DHS 2, respectively (see Figure 5.4, panel A). Probe B was used to detect DHS 3; this probe detects a 20kb genomic fragment including the fourth and fifth exons. Detection of an additional 3.6kb band in digests of
DNA prepared from DNase I treated nuclei indicated the presence of DHS 3 (Figure 5.4, panel B). In samples lacking detectable hypersensitive sites, efficacy of DNase I and restriction enzyme digestions was confirmed by rehybridisation of filters to probes designed to detect constitutive hypersensitive sites within the α-globin promoter and upstream regulatory element, HS -40. For Bam HI digests, probe \( \psi \alpha \) was used, detecting constitutive hypersensitive sites at coordinates +17 and +22 within the α-globin promoter region (Craddock et al., 1995) as shown in Figure 5.4 (panel E); in HEL and K562 cell lines additional erythroid specific sites at coordinates +20 and +24 were detected (Figure 5.6, panel E). For EcoRI digests, probe L1 was used, detecting a hypersensitive site relating to a gene with a Cpg island at coordinate -14, as shown in Figure 5.4 (panel F). L1 and \( \psi \alpha \) probes were generated by PCR from PAC 7172 encompassing the α-globin upstream regulatory elements (generously provided by Prof.D.R.Higgs, Institute of Molecular Medicine, Oxford), using the following primers:

- L1 forward: 5'-TTATCGGTCTGCTCCACTTGGTCTG-3',
- L1 reverse: 5'-ATGCTGTTGTCACACTGGTGTTTC-3'
- \( \psi \alpha \) forward: 5'-TTTGGCAGGACTAAGAGACGCAG-3',
- \( \psi \alpha \) reverse: 5'-GAGGCACCACATGGGTAAACTG-3'
Detection of DNase I hypersensitive sites in the 5' and 3' flanking regions of the CD2 gene

Figure 5.3
Position of DNase I hypersensitive sites relative to the genomic structure of the CD2 gene as defined in peripheral blood T-lymphocytes and Jurkat cells (Wotton et al, 1989). 5' hypersensitive sites lie within the promoter (DHS 2) and upstream regulatory elements (DHS 1), whereas DHS 3 lies within the 3' enhancer region. "B" and "R" denote Bam HI and Eco RI restriction sites respectively. Black boxes denote exons. Also shown is the position of cDNA probes A and B (denoted by grey boxes) used for hybridisation to Southern blots, in relation to the genomic structure.
A) Detection of DNase I hypersensitive sites in the 5' flanking region of CD2 in CD2 positive peripheral T cells, demonstrating presence of DHS 1 & 2. In this examination the concentrations of DNase I used favoured detection of DHS 2.

B) Detection of DNase I hypersensitive sites in the 3' flanking region of CD2, demonstrating the presence of DHS 3.

In the CD2 negative B cell line Daudi, 5' (C) and 3' (D) flanking regions of CD2 were inaccessible to DNase I. Filters were rehybridised with probes derived from the α-globin upstream regulatory region, demonstrating constitutive hypersensitive sites, as shown in panels E and F.

Figure 5.4 Detection of DNase I hypersensitive sites in the 5' and 3' flanking regions of the CD2 gene in T and B lymphoid cells.

In each case increasing amounts of DNase I are denoted by the triangle above the autoradiograph. Bands indicating the presence of hypersensitive sites are denoted by the arrowheads. * indicates a band for which the origin is not certain.
Figure 5.5 Detection of DNase I hypersensitive sites in the 5' and 3' flanking regions of the CD2 gene, in CD2 positive and negative cases of hypogranular variant APL

In each case increasing amounts of DNase I are denoted by the triangle above the autoradiograph. Hypersensitive sites are denoted by arrowheads. * indicates a band for which the origin is not certain.

CD2 positive APL

A) Detection of DNase I hypersensitive sites in the 5' flanking region of CD2, demonstrating presence of DHS 1 & 2.
B) Detection of DNase I hypersensitive sites in the 3' flanking region of CD2, demonstrating presence of DHS 3.

CD2 negative M3v

C) Detection of DNase I hypersensitive sites in the 5' flanking region of CD2, demonstrating presence of DHS 1 & 2.
D) Detection of DNase I hypersensitive sites in the 3' flanking region of CD2, demonstrating presence of DHS 3.

In leukaemic cells derived from a patient with B lineage PLL, 5' (E) and 3' (F) flanking regions of CD2 were inaccessible to DNase I.
Filters were rehybridised with probes derived from the α-globin upstream regulatory region, demonstrating the presence of constitutive hypersensitive sites, as shown in panels G & H.

DHS -14

DHS +22

DHS +17
Figure 5.6 Detection of DNase I hypersensitive sites in the 5' and 3' flanking regions of the CD2 gene in CD2 negative U937 and HEL cell lines. In each case increasing amounts of DNase I are denoted by the triangle above the autoradiograph. Bands indicating the presence of hypersensitive sites are denoted by arrowheads. * indicates a band for which the origin is not certain.
Results of DNase I hypersensitivity assays performed in the Jurkat cell line and normal donor peripheral T lymphocytes cultured in IL-2 and PHA which are both CD2 positive, and in B lineage Daudi cells which lack surface expression of CD2 (see Table 5.1), served as positive and negative controls respectively. All three sites were detected in Jurkat and peripheral T cells (see Table 5.3; Figure 5.4 panels A, B), whilst chromatin surrounding 5' and 3' flanking regions was found to be inaccessible to DNase I in Daudi cells (Figure 5.4, panels C, D), in accordance with previous studies (Wotton et al., 1989). Analysis of leukaemic blasts derived from 6 patients with CD2 positive APL, revealed an identical pattern of hypersensitive sites to that detected in normal T lymphocytes and Jurkat cells (e.g. Case P3, shown in Figure 5.5 panels A, B). Similarly, all three sites were detected in both patients with variant M3 who were found to lack surface expression of CD2 (e.g. Case P7, Figure 5.5 panels C, D). In all of these cases, immunophenotype analysis of mononuclear cells used for DNase I hypersensitivity assays revealed that T lymphocytes comprised <2%, suggesting that hypersensitive sites detected were a true reflection of the chromatin structure surrounding CD2 in the leukaemic population, rather than an effect due to contaminating lymphocytes. This was confirmed by analysis of a chronic B cell neoplasm (P12), prolymphocytic leukaemia (PLL), revealing a closed chromatin pattern (see Figure 5.5, panels E, F), identical to that detected in the Daudi cell line.

In order to determine whether an open chromatin configuration surrounding 5' and 3' flanking regions of CD2 was specific to the hypogranular variant form of APL, the APL cell line NB4 derived from a patient with classical APL (Lanotte et al., 1991) and a series of other myeloid cell lines, including HL60 (AML M2, Dalton et al., 1988), U937 (promonocyte), HEL (human erythroleukaemia) and K562 (CML), were evaluated; examination of primary material from classical APL cases was precluded by insufficient cell numbers. Each of the cell lines analysed were found to lack surface expression of CD2 as determined by flow cytometry (Table 5.1). Nevertheless, all hypersensitive sites were present in NB4, HL60 and U937 cells (e.g U937 shown in Figure 5.6, panels A, B), although bands associated with DHS 1 and DHS 2 were weak in NB4. In HEL, only DHS 3 and a weak band corresponding to DHS 2 were detected (Figure 5.6, panels C, D); whilst all regions of CD2 were found to be inaccessible to DNase I in K562 cells, as previously shown for the Daudi cell line (Figure 5.4, panels C, D).
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Cell lines/ peripheral T cells

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Table 5.3

Results of DNase I hypersensitivity assays performed in the hypogranular variant form of APL (M3v), B prolymphocytic leukaemia (B-PLL) and haemopoietic cell lines. Analyses undertaken in cases P6, 10, 11 & 13 were excluded due to excess T cell contamination (≥5%) or unsatisfactory α-globin probe controls.

+/-, indicates that the band associated with a particular hypersensitive site appeared weak.
Expression of CD2 in haemopoietic cell lines

Figure 5.7

Determination of CD2 expression in haemopoietic cell lines by Northern analysis. Relative loading of total RNA is indicated by corresponding ethidium bromide stained gel shown below.
In order to correlate chromatin structure with gene transcription, Northern analyses were performed using total RNA derived from selected cell lines (Figure 5.7). The CD2 cDNA probe used was derived by nested RT-PCR using the following primers:

first round,
forward 5'-TTGTAGCCAGCTTCCTTCTG-3',
reverse 5'-TGGGAAGTTGCTGGATTCTG-3';

second round,
forward 5'-GCATCTGAAGACCGATGATC-3',
reverse 5'-CTTCAGTAGCTACTCTGTGG-3'.

Specificity of the PCR product was confirmed by sequence analysis prior to use. As expected, high level CD2 expression by the Jurkat T cell line was evidenced by the presence of 1.7 and 1.3kb bands as previously reported (Sewell et al., 1986). Interestingly, a weak 1.7kb band was also detected in the other cell lines tested, including the B lymphoid line Daudi. This result would suggest that expression of CD2 is not completely extinguished on differentiation along myeloid and B lineage pathways. Indeed nested RT-PCR confirmed expression of CD2 transcripts in each of the cell lines that were examined by Northern analysis, including Daudi (data not shown). The discrepancy between these results and those obtained by flow cytometry (Table 5.1), could be accounted for lack of externalisation of antigen in the myeloid and B cell lines tested, or alternatively if CD2 is expressed at the cell surface, antigen levels may fall below the level of sensitivity of immunophenotype analysis.

5.3 Implications of results of DNase I hypersensitivity assays

The results of the DNase I hypersensitivity assays presented in this chapter raise a number of interesting issues, enabling one to more specifically address the mechanisms underlying CD2 expression in normal haemopoietic differentiation and in myeloid leukaemias such as APL. In particular, this study has confirmed that in common with CD2 expressing Jurkat cells and peripheral blood T lymphocytes, 5' and 3' regulatory regions of CD2 lie within an open chromatin configuration in CD2 positive cases of APL. Interestingly, an identical pattern of hypersensitive sites was identified in the remaining cases of M3v and in the APL (NB4), AML M2 (HL60) and promonocyte (U937) cell lines, which were all found to lack surface expression of CD2 by flow cytometry. Previous studies performed solely in transformed cell lines and normal peripheral T lymphocytes had suggested that the regulatory regions 5' to CD2 play a key role in determining stage specific expression of the gene, on the basis that DHS 1 and DHS 2 appeared to be restricted to CD2 expressing T cells (Wotton et al., 1989).
However, these studies did not include myeloid lineages. The present study clearly demonstrates the presence of DHS 1 and 2 in a series of myeloid cell lines which do not express CD2 at high level. These findings would imply that additional factors, that are independent of nucleosomal displacement at regulatory elements are key determinants of the relative level of CD2 expression. Whilst it is possible that such effects are mediated at 3' and/or 5' sites, recent studies identifying activator and silencer activities in the region of DHS 1 provide a potential mechanism for regulation of gene expression through the interaction of transcription factors binding at this site.

Analysis of the remaining myeloid cell lines which also lacked detectable surface CD2, revealed the presence of DHS 3 and a weak DHS 2 associated band in the human erythroleukaemia cell line HEL, whilst all sites were inaccessible to DNase I in K562 cells, akin to the pattern demonstrated by B cell lines. HEL and K562 both have some degree of erythroid differentiation as evidenced by globin gene expression. Hence, these findings raise the possibility that differentiation along B lymphoid or erythroid lineages could be associated with closing down of chromatin surrounding CD2, whilst differentiation along myeloid pathways may be associated with a persistently open pattern, similar to that detected in normal T lymphocytes. In such a scheme, high level expression of CD2 associated with mature T cells could be mediated through expression of T cell specific factors. Possible mechanisms accounting for the expression of T lineage antigens such as CD2 in myeloid malignancies and the implications of this phenomenon to the pathogenesis of APL are considered in the following chapter.
This thesis sought to address three main issues pertinent to the biology of APL and treatment of patients with this disease, namely 1) the role of molecular techniques as a means of confirming the diagnosis and providing prognostic information, 2) evaluation of nested RT-PCR for the detection of minimal residual disease, as an indicator of response to therapy and subsequent outcome, and 3) the mechanisms underlying expression of the T-lineage antigen CD2 in this condition. In addition to suggesting that molecular diagnosis and monitoring using RT-PCR could be of value as a means of determining treatment approach for patients with APL, these studies have provided some further insights into the pathogenesis of the disease. This chapter will describe the major findings of this thesis, consider their implications to the molecular mechanisms leading to the development of APL and suggest directions for future study arising from this work.

6.1 Role of molecular diagnosis in APL

Early studies performed in specialist cytogenetic centres had suggested that the t(15;17) could be detected in all cases of APL (Rowley et al., 1977; Larson et al., 1984). In the light of such claims, clinicians encountering cases of AML with morphological features of APL could potentially doubt the initial clinical diagnosis if subsequent cytogenetic assessment failed to provide appropriate confirmatory evidence. However, since the characterisation of cases involving cryptic PML/RARα rearrangements (Borrow et al., 1994; Hiorns et al., 1994; Lafage-Pochitaloff et al., 1995) and identification of the rare alternative translocations whereby RARα is fused to partners other than PML (Chen et al., 1993a,b; Redner et al., 1996; Wells et al., 1997) it is clear that absence of the t(15;17) does not preclude a morphologic diagnosis of APL. Establishing the nature of the underlying molecular abnormality has a fundamental bearing on the optimal treatment approach for these disease entities, due to their differential sensitivity to retinoid differentiating agents. Multi-centre APL trials have established that combination therapy using ATRA in conjunction with chemotherapy, confers a significant survival advantage due to a reduced risk of relapse compared to chemotherapy alone (Fenaux et al., 1994; Kanamaru et al., 1995; Tallman et al., 1997; Burnett et al., 1997). It is now clear that the presence of PML-RARα (Miller et al., 1992; Mozziconacci et al., 1998),
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*NPM-RARα* (Redner et al., 1997) and possibly *NuMA-RARα* fusions (Wells et al., 1997) predicts a favourable response to retinoids, suggesting that patients with these forms of APL should not be denied retinoid therapy, which could improve their chances of cure. Conversely, previous studies have found APL cases with *PLZF/RARα* rearrangements to be resistant to ATRA and its use as single agent therapy in this group has been associated with a poor prognosis (Licht et al., 1995), suggesting that chemotherapy should be included in the induction treatment protocol of such patients. Adoption of a tailored treatment approach directed by the underlying molecular lesion, is entirely dependent upon successful disease characterisation at diagnosis. However, no studies had previously specifically determined the reliability of conventional cytogenetic analysis to identify the presence of underlying *PML/RARα* rearrangements through the detection of the t(15;17), nor had they established the frequency of cryptic *PML/RARα* rearrangements or alternative APL associated translocations. Molecular analysis of a large group of patients entered into the MRC ATRA trial afforded the opportunity to resolve these issues. Overall, amongst 187 APL cases analysed by nested RT-PCR, over 99% (186/187) were found to have an underlying *PML/RARα* rearrangement, whilst in the remaining case *PLZF-RARα* and *RARα-PLZF* fusion transcripts were detected associated with the t(11;17)(q23;q12-21) cytogenetic abnormality. Comparison of results obtained by molecular screening and conventional cytogenetic analysis, revealed that RT-PCR successfully identified *PML/RARα* rearrangements in all patients with documented t(15;17) or 3-way translocations involving chromosomes 15 and 17, but additionally revealed underlying *PML/RARα* rearrangements in a further 15% patients (28/186) lacking the t(15;17). This latter phenomenon highlighted a number of shortcomings of cytogenetic analysis. In some instances absence of the t(15;17) reflected failure of cytogenetic cultures or the presence of poor quality metaphases, whilst in others reported to have a normal karyotype *PML-RARα* and *RARα-PML* fusion transcripts were both detected by RT-PCR, suggesting that the t(15;17) had been present but had been missed despite the use of prolonged culture techniques. Interestingly, in the remaining patients who were found to have a cytogenetic changes other than the t(15;17) or a normal karyotype, *PML-RARα* was the sole fusion transcript detected, consistent with the occurrence of cryptic rearrangements due to insertion events. This was subsequently confirmed by FISH analyses performed in 4 such patients with evaluable metaphase spreads. In 3 cases *RARα* was inserted into *PML* on 15q, in accordance with two previous case reports in which chromosomes 15 and 17 also appeared normal by conventional cytogenetics (Hiorns et al., 1994; Lafage-Pochitaloff et al., 1995); whilst in the remaining patient the *PML-RARα* fusion resulted from insertion of *PML* into *RARα* on 17q, which has not been previously described.
Demonstration of PML-RARα as the sole fusion gene formed in each of the APL cases with cryptic PML/RARα rearrangements in this study is consistent with the proposed role of its gene product as a critical mediator of leukaemogenesis. This has recently been confirmed in a transgenic model whereby expression of PML-RARα was associated with impairment of normal myeloid differentiation accompanied by accumulation of primitive precursors and predisposition to an APL-like syndrome responsive to ATRA (Brown et al., 1997; Grisolano et al., 1997; He et al., 1997). Although there was noted to be a latent period prior to onset of the leukaemia arguing in favour of a requirement for additional mutational events, as has been suggested in theoretical models of tumourigenesis (Vickers, 1996). This prompted the suggestion that the reciprocal RARα-PML fusion product could play a role in this process (Pandolfi, 1996; He et al., 1997), particularly following reports of a case of APL in which RARα-PML was apparently the sole fusion gene formed (Lafage-Pochitaloff et al., 1995; Mozziconacci et al., 1998). However, the observations that not all PML-RARα mice developed APL, or did so after a latent period could also imply that high-level expression of the transgene did not occur within equivalent progenitors to those forming targets of leukaemic transformation in human APL. Indeed, a number of findings of this thesis would suggest that RARα-PML is unlikely to contribute to the pathogenesis of APL. In particular RARα-PML was only found to be expressed in 76% cases; furthermore, no correlation was observed between the presence of RARα-PML transcripts and a variety of disease characteristics, including presenting WBC or outcome (Table 3.1).

In view of the high risk of early mortality resulting from haemorrhage which is a frequent complication of APL (Tallman & Kwaan, 1992), rapid diagnosis of this condition is essential. Due to the necessary culture steps, cytogenetic analyses require several days to perform in order to provide reliable results, and hence are not suitable as a means of rapidly confirming a clinical diagnosis of APL. Nested RT-PCR, as described in this thesis would take a minimum of 2 working days to perform; although shortening of RNA extraction and reverse transcription steps, with a single round of PCR can produce reliable results within 1 day (Diverio et al., 1996). The demonstration in this thesis, that virtually all patients with APL have an underlying PML/RARα rearrangement, highlights the potential merit of the PML-immunofluorescence technique, as a rapid diagnostic test for APL. In the presence of the PML-RARα fusion protein, PML monoclonal or polyclonal antibodies detect a characteristic microparticulate nuclear staining pattern, indicative of disruption of PML nuclear bodies (Daniel et al., 1993; Dyck et al., 1994; Weis et al., 1994; Koken et al., 1994).
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previous study had evaluated this technique in cytospin preparations (Dyck et al., 1995); however, in this thesis it was confirmed that buffy coat preparations derived from bone marrow or blood (provided leukaemic blasts are present) are also suitable. It is clear that for reliable results it is essential to exclude cellular disruption, either by nuclear counter-staining or by use of phase contrast. PML-immunofluorescence can be performed within 4 hours, confirming its suitability as a rapid diagnostic test for APL; indeed this has since become standard practice in some clinical centres (O'Connor et al., 1997). In addition, the availability of material derived from the case of APL with the t(11;17) with a confirmed PLZF/RARα rearrangement, afforded the opportunity to use immunofluorescence techniques to address more fundamental issues relating to the pathogenesis of APL. The discovery that PML nuclear bodies are disrupted in APL, prompted the suggestion that this process is critical to the pathogenesis of the disease and that reconstruction of the normal nuclear architecture is critical to the differentiation response to ATRA (Dyck et al., 1994). The characterisation of PLZF/RARα, NPM/RARα and NuMA/RARα rearrangements which are also associated with morphological APL, provided a chance to test this hypothesis. Interestingly PLZF and PML both possess growth suppressor activity; furthermore, the proteins have been shown to interact, and colocalise within PML nuclear bodies (Pandolfi, 1996; Koken et al., 1997; Shakhnovich et al., 1998). This raised the possibilities that their growth suppressor activities might be linked, and that disruption of PML nuclear bodies could provide the final common pathway to the pathogenesis of APL. However in the present study, immunofluorescence analyses in the case with the PLZF-RARα fusion demonstrated localisation of PML within discrete nuclear bodies. This would indicate that disruption of PML nuclear bodies is unlikely to be critical to the pathogenesis of APL. This is supported by recent studies which have also demonstrated a wild type PML nuclear staining pattern in NPM-RARα (Redner et al., 1997) and NuMA-RARα (Wells et al., 1997) associated APL. Furthermore, studies involving expression of a variety of PML-RARα mutants in haemopoietic cell lines have established that the differentiation block mediated by the fusion protein is independent of PML delocalisation (Grignani et al., 1996). Nevertheless, it remains a possibility that nuclear body constituents could play an important role in leukaemic transformation in all forms of APL. In this regard, it is interesting that recent co-transfection studies have demonstrated that PML-RARα and PLZF-RARα colocalise with PLZF within microspeckled structures (Koken et al., 1997; Ruthardt et al., 1998). Characterisation of components within these structures may lead to further advances in understanding of the pathogenesis of all forms of APL. It will be of great interest to determine whether deregulation of the growth suppressor PLZF,
compounded by a differentiation block mediated by the chimaeric retinoic acid receptor is indeed the final common pathway to the pathogenesis of this disease.

Molecular screening of a large group of APL patients using RT-PCR also afforded the opportunity to determine whether PML breakpoint is indeed associated with a variety of disease characteristics, as has been reported by a number of previous smaller studies. In particular, a correlation between the bcr 3 breakpoint pattern and the presence of additional cytogenetic abnormalities (Slack et al., 1997) was not confirmed. However, an increased risk of relapse was noted in patients with this breakpoint pattern (Table 3.1, Figure 3.11), in accordance with some previous reports (Borrow et al., 1992; Vahdat et al., 1994), although this effect did not reach statistical significance and was not associated with any difference in overall survival (Figure 3.12). Another large study has not confirmed PML breakpoint as an independent prognostic factor, and suggested that any adverse prognostic influence of the bcr 3 breakpoint could be accounted for by an association with high presenting WBC (Gallagher et al., 1997). It is possible that the conflicting data reported could be a reflection of relatively small sample sizes, inter- and intra-study treatment variability, compounded by the relatively low relapse rate observed in APL. Resolution of this issue will require study of even larger numbers of identically treated patients. Certainly, at present there is insufficient evidence to support PML breakpoint pattern as a basis to determine treatment approach. Should PML breakpoint ultimately be shown to have a significant bearing on prognosis, it will be of interest to determine the molecular basis of this phenomenon. Recent studies have suggested some functional differences between bcr 1, bcr 2 and bcr 3 PML-RARα fusion proteins (Gallagher et al., 1995; Slack & Yu, 1998), raising the possibility that such effects could reflect differential interactions with factors such as PIC1 and nuclear corepressors or coactivators.

It is of interest that a number of aspects of gene rearrangements in APL, bear some similarities to those previously described in chronic myeloid leukaemia (CML). The latter disease is characterised by the t(9;22), leading to the formation of BCR-ABL and reciprocal ABL-BCR fusion genes at 22q11 and 9q34 respectively (reviewed Hagemeijer, 1987; Melo et al., 1993). In common with PML-RARα, BCR-ABL has been considered the key oncogenic fusion protein, associated with development of leukaemia in transgenic models (Heisterkamp et al., 1990). Interestingly, approximately 3% CML cases are associated with cryptic gene rearrangements (Hagemeijer, 1987); in accordance with the frequency of this phenomenon in APL, as determined by this thesis. Furthermore, in the majority of these cases the BCR-ABL (Morris et al., 1986, 1990;
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Ganesan et al., 1986; Dreazen et al., 1987; Inazawa et al., 1989; Rassool et al., 1990) and PML-RARα fusion genes (Brunel et al., 1996) are found at their typical locations of 22q11 and 15q22, respectively. This would suggest that similar processes may be involved in mediating leukaemogenic gene rearrangements and chromosomal translocations in these diseases. Two models have been proposed to account for cryptic gene rearrangements. The first, postulates a two stage mechanism whereby the classical translocation is formed initially, followed by a further reciprocal translocation event reconstituting chromosomes of normal appearance, but leaving the fusion gene at its typical location (Morris et al., 1986; 1990). This hypothesis can readily account for cryptic rearrangements in which BCR-ABL and PML-RARα fusions are formed at their usual locations. However, these findings are also compatible with the alternative model, whereby cryptic gene fusions result from non-reciprocal insertion events, rather than chromosomal translocations (Rassool et al., 1990; Hiorns et al., 1994). This latter mechanism is more easily reconciled with reports of BCR-ABL and PML-RARα fusions identified at chromosomal sites typically occupied by the respective reciprocal fusion genes (Hagemeijer et al., 1993; Grimwade et al., 1997). Characterisation of genomic regions flanking complex translocation breakpoints in patients with CML has already provided some insights into the mechanisms underlying chromosomal rearrangements. (Sowerby et al., 1993). It is likely that similar studies undertaken in APL cases with cryptic or complex rearrangements could be worthwhile. There is accumulating evidence for clustering of breakpoints in this disease within the second intron of RARα, which extends for at least 10kb. This is supported by the finding in this thesis of identical stretches of RARα intron-derived nucleotides at PML/RARα bcr 2 cDNA fusion junctions in 3/10 cases analysed, which were also found to match the junctional nucleotides of a previously reported case (Pandolfi et al., 1992). Further evidence for breakpoint clustering has been provided by detailed analysis of cases of secondary APL arising following etoposide therapy (Kudo et al., 1998). It is hoped that further characterisation of the genomic regions surrounding PML and RARα breakpoints may provide further insights into the mechanisms underlying the development of the t(15;17) and hence APL.

A further aspect of the present study was the description of the t(15;17) with formation of the PML-RARα fusion gene in two cases of AML lacking typical APL morphology, who were not included in the MRC trials. This is in accordance with recent reports (Neame et al., 1997; Foley et al., 1998; Aventin et al., 1998) and is of interest for a number of reasons. First, it demonstrates that the t(15;17) is not restricted to cases with M3 morphology contrary to previous dogma. Secondly, it confirms the link between
expression of the PML-RARα fusion protein and disruption of nuclear bodies and the response to ATRA, highlighting the merits of the PML-immunofluorescence technique as a means of identifying patients with AML most likely to benefit from retinoids. As to why, these two cases lacked the accumulation of abnormal promyelocytes that characterises APL could have a number of explanations. It is possible that the PML/RARα rearrangement targeted a different haemopoietic progenitor in terms of lineage commitment and/or differentiation potential from that typically associated with APL. Alternatively, characteristic promyelocytic features may have been present initially, but lost due to the predominance of a population of blasts acquiring an additional event, associated with clonal evolution leading to a more proximal block in myeloid differentiation. As previously mentioned, transgenic studies have suggested that the PML-RARα fusion protein is insufficient to mediate APL and that additional oncogenic events are required. If this is indeed so, such events do not preclude the development of promyelocytic features in the majority of cases, but it remains a possibility that occasionally such events could lead to a more proximal differentiation block thereby preventing the accumulation of abnormal promyelocytes. However, this latter hypothesis appears to be at variance with the retinoid responsiveness of these forms of AML. This raises a further possibility, that the morphological appearances of the bone marrow in these atypical cases actually reflect an influence of the bone marrow microenvironment rather than any intrinsic characteristic of the leukaemic population per se. In support of this view it should be noted that blasts from one of the cases described here appeared significantly more granular after 5 days in culture, being more reminiscent of M3variant cells. The influence of external stimuli on APL morphology has previously been observed (Berger et al., 1981). The role of an abnormal stromal microenvironment is becoming increasingly recognised as an influential factor in tumourigenesis (Kinzler & Vogelstein, 1998).

The identification of patients with the t(15;17) but lacking APL morphology, raised the issue that molecular screening for the PML/RARα rearrangement should be performed in patients with newly diagnosed AML (Neame et al., 1997). A recent study which used a multiplex PCR approach to detect a wide range of translocation fusion transcripts appeared to support this view (Pallisgaard et al., 1998). However, it should be noted that no morphological review was included in the latter study; raising the possibility that screening merely served to identify cases of APL who could have been distinguished on morphological grounds alone, and that absence of the t(15;17) in these cases reflected cytogenetic failures or insertion events as described in this thesis. Indeed, we have recently shown that molecular screening for the PML/RARα rearrangement in other
subtypes of AML is unlikely to be worthwhile; screening of material derived from 530 MRC trial patients by RT-PCR for PML-RARα and RARα-PML transcripts, revealed only 1 positive case, which subsequently transpired to be a missed example of the hypogranular variant form of APL (Langabeer et al., 1998; Allford et al., 1998 submitted). This would suggest that molecular analyses are best restricted to patients with morphological M3/M3v, to cases of acute leukaemia with some morphological features of APL (even if occurring in a minority of cells), and to cases of acute leukaemia associated with an APL-like immunophenotype (CD9+, CD13+, CD33+, HLA-DR-) or with a severe coagulopathy.

6.2 Role of molecular monitoring in APL

This thesis also sought to evaluate the role of molecular monitoring for minimal residual disease (MRD) in APL, using nested RT-PCR assays to detect PML-RARα and RARα-PML fusion transcripts. Whilst a number of early studies of MRD monitoring in this disease implied that PCR status at the end of therapy is a major determinant of outcome (Lo Coco et al., 1992; Miller et al., 1992); a key finding of the present study is the demonstration that MRD analysis performed immediately following completion of consolidation therapy is not a reliable prognostic indicator in patients treated with current standard treatment protocols involving combinations of ATRA and chemotherapy. This has recently been supported by data derived from the Italian AIDA study (Diverio et al., 1998), and reflects the relative insensitivity of the assays used. Indeed, recent analyses using a quantitative RT-PCR approach would suggest that this is largely accounted for by the low level of fusion gene expression by APL blasts (Seale et al., 1996). Since the RARα-PML assay has been found by our laboratory (Linch et al., 1994) and others (Tobal et al., 1995) to be more sensitive than the more conventional PML-RARα assay, it was of interest to determine whether monitoring for both transcripts could enhance MRD detection and identify the majority of patients at risk from relapse. Whilst the RARα-PML assay did increase detection of residual disease in patients in morphological CR during the consolidation phase of therapy, it failed to identify the majority of patients who ultimately relapsed.

Having established that molecular monitoring performed immediately following completion of therapy is of limited prognostic value, it was of interest to determine whether the rate of disappearance of fusion transcripts during consolidation therapy was more predictive of outcome. Interestingly, the detection of residual disease at any stage during therapy was associated with an increased risk of relapse (Table 4.1); this was
found to be most predictive following the third course of chemotherapy when most patients were evaluable, corresponding with the timing of bone marrow harvesting. Detection of MRD at this stage predicted an increased risk of relapse associated with a poorer overall survival (Figures 4.4, 4.5). This result should be confirmed in a larger group of patients treated with chemotherapy and extended courses of ATRA. Nevertheless, these data are in accordance with previous MRD monitoring studies using flow cytometric or PCR-based methods which have suggested that the rate of disappearance of the disease-related clone is predictive of outcome (Brisco et al., 1994; San Miguel et al., 1998; Coustan-Smith et al., 1998; Cavé et al., 1998). However, an advantage of the present study is that it suggests that molecular monitoring can be used to identify groups of patients at high and low risk of relapse in the context of a relatively homogeneous disease. This is particularly pertinent in APL, since this subtype of AML has been found to have a relatively favourable prognosis, such that routine application of bone marrow transplantation in first remission does not confer any overall survival benefit (Burnett et al., 1998). Therefore, it becomes increasingly important to identify groups of patients at high risk from relapse who could benefit from further consolidation therapy. A recent study has also suggested that molecular monitoring for the PML-RARα fusion is highly predictive of subsequent relapse when sequential tests are performed after completion of therapy (Diverio et al., 1998). Since frank haematological relapse in APL is associated with a poor prognosis, these data would suggest a novel treatment approach whereby therapy is recommenced much earlier, i.e. at the point of molecular relapse. Molecular monitoring studies in CML post BMT have already set a precedent for this strategy, demonstrating that treatment of patients at the point of molecular relapse using donor lymphocyte infusions is associated with a significantly better outcome, than treatment at the point of haematologic relapse (Van Rhee et al., 1994). In the light of a recent study which has documented the feasibility of ATRA therapy as a means of achieving long term remission in APL patients with residual disease following ABMT (Grimwade et al., 1998b), retinoid therapy alone could be sufficient for the treatment of minimal residual disease detected following completion of consolidation therapy (subject to continued molecular monitoring). However, it is clear that the success of this approach is dependent upon detecting cases with acquired mutations within the PML-RARα fusion gene (Imaizumi et al., 1998; Ding et al., 1998) which would require treatment with chemotherapy, or novel agents such as arsenic compounds. It will be of great interest over the next few years to determine whether MRD detection during or after consolidation therapy using novel techniques such as "real-time" quantitative PCR (Marcucci et al., 1998) will afford greater reliability in distinguishing
patients destined to remain in long term remission from those who ultimately relapse, thereby enabling precise modification of treatment according to molecular response.

Therefore, this thesis supports a role for routine use of nested RT-PCR to detect the PML/RARα rearrangement in all patients with suspected APL, both as a means of identifying the subgroup of patients likely to benefit from retinoid therapy, and also to define targets for subsequent minimal residual disease monitoring. Molecular screening of patients entered into the MRC ATRA trial has established that the group with PML/RARα rearrangements documented solely by molecular techniques share the relatively favourable prognosis of those with the t(15;17) identified by cytogenetics (Figure 3.13). This suggests that they represent the same disease entity, and indeed recent studies have confirmed that APL cases with cryptic PML-RARA fusions respond to ATRA in vitro (Mozziconacci et al., 1998). This is of particular interest, since it would indicate that patients with AML in whom molecular screening studies have identified cryptic AML1-ETO (Andrieu et al., 1996; Langabeer et al., 1997a) or MYH11-CBFβ (Poirel et al., 1995; Langabeer et al., 1997b) fusions could share the favourable prognosis, previously associated with patients with their respective t(8;21) or inv(16) chromosomal rearrangements (Dastugue et al., 1995; Grimwade et al., 1998a). These subtypes of AML also require a specific treatment approach, since in common with APL, BMT in first remission has been shown to confer no overall benefit in this group (Burnett et al., 1998). The importance of molecular characterisation of AML as a means of determining future therapeutic approaches has been particularly highlighted by recent studies addressing the mechanisms underlying the differentiation block and ATRA response in AML. The PML-RARα fusion protein was shown, not only to be critical for leukaemogenesis through the recruitment of corepressors and HDAC to retinoid response elements, but also to mediate the clinical response to ATRA (Lin et al., 1998; Grignani et al., 1998; Guidez et al., 1998; He et al., 1998). In addition it was shown that retinoid resistance in PLZF-RARα mediated APL could be overcome by HDAC inhibitors (He et al., 1998), raising the possibility that retinoids in combination with agents such as butyrates could provide a significant therapeutic advance in this disease in the future. A recent report demonstrated that the corepressor complex can also bind to ETO (Wang et al., 1998a). This suggests a common theme in haematological malignancy whereby chromosomal rearrangements generate chimaeric transcription factors, which target the corepressor/HDAC complex to regulatory elements of genes playing a key role in haemopoietic differentiation. Further understanding of the mechanisms regulating chromatin structure and the remodelling mediated by oncogenic fusion gene products could lead to important advances in the treatment of
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haematological malignancies. This raises the exciting possibility that in the future treatment of AML will increasingly be directed towards the treatment of molecularly defined subgroups, with therapeutic approach being further tailored according to response as determined by monitoring for MRD. APL has already provided an exciting model for this process.

6.3 Implications of CD2 expression in APL

A further aspect of this thesis related to investigation of the mechanisms underlying the expression of the T-lineage marker CD2 in APL. Recent studies have shown that this phenomenon is largely restricted to the hypogranular variant form of the disease (Biondi et al., 1995; Guglielmi et al., 1998). For many years it has been debated as to whether the expression of inappropriate surface antigens indicates that such leukaemias represent expansions of rare stem cells that co-express lymphoid and myeloid antigens or alternatively arise in lineage-committed cells in which the presence of "inappropriate" surface markers reflects aberrant gene expression associated with leukaemogenesis (Greaves et al., 1986; Smith et al., 1983). In order to begin to address this issue, DNase I hypersensitivity assays were performed to determine the chromatin structure surrounding the 5' and 3' flanking regions of CD2, in blasts derived from a series of CD2 positive and negative M3v cases and in haemopoietic cell lines. Previous analyses had confirmed that all key regulatory elements determining expression of CD2 are contained within this region (Lang et al., 1988; Greaves et al., 1989) and were subsequently identified by the presence of DNase I hypersensitive sites (Wotton et al., 1989). In CD2 positive Jurkat cells two hypersensitive sites were detected in the 5' flanking region denoted DHS 1 and 2; DHS 1 has since been shown to lie in a region with a silencer element, and an activator element which is necessary for high level expression of CD2, whilst DHS 2 lies adjacent to the transcription start site, corresponding to the core promoter (Wotton et al., 1989; Outram et al., 1994; Outram, 1996). The 3' flanking region contains further hypersensitive sites, denoted DHS 3, corresponding to enhancer elements and an LCR (Wotton et al., 1989; Greaves et al., 1989; Festenstein et al., 1996). Previous studies using transgenic mice had suggested that the enhancer elements and LCR play a key role in directing tissue specific expression of CD2 (Greaves et al., 1989). In support of this, DNase I hypersensitivity assays performed in a variety of lymphoid lines, reported that DHS 3 was restricted to cells of T cell lineage. Furthermore, DHS 1 and DHS 2 were detected solely in cell lines expressing CD2 at the cell surface, implicating these regions as important determinants of stage-specific
expression of CD2 (Wotton et al., 1989). However, the chromatin structure surrounding the CD2 locus had not previously been determined in the myeloid lineage. This thesis revealed an identical pattern of hypersensitive sites in CD2 positive cases of APL to that previously reported in T lineage Jurkat cells and peripheral blood lymphocytes (Wotton et al., 1989; Outram, 1996). Interestingly, all three hypersensitive sites were also detectable in blasts derived from CD2 negative cases of APL and the myeloid leukaemia cell lines NB4, HL60, and U937. Since previous studies have established a close relationship between detection of CD2 by flow cytometry and the level of gene expression as determined by Northern analysis, both in haemopoietic cell lines and primary APL blasts (Wotton et al., 1989; Biondi et al., 1995); these findings would suggest that additional factors, that are independent of nucleosomal displacement at regulatory elements are key determinants of the relative level of CD2 expression.

Recent studies have characterised a number of putative transcription factor binding sites in the upstream regulatory region of CD2 whose integrity is critical for high level gene expression (Outram et al., 1994; Outram, 1996). In the DHS 2 promoter region these include a GATA site (possibly binding GATA-3) and an E box, shown to bind the helix-loop-helix protein USF1 (Outram et al., 1994). Interestingly, the E box motif can also bind Myc, Max and Mad proteins (Kretzner et al., 1992; Blackwood et al., 1992; Gu et al., 1993; Ayer et al., 1993), which have themselves been proposed to be deregulated in APL due to sequestration of Mad/Max by the fusion protein/corepressor complex (He et al., 1998; Guidez et al., 1998). Binding of Max to the E box has been found to repress CD2 expression; whilst USF1 leads to transcriptional activation (Outram et al., 1994), suggesting that differential transcription factor binding at this element could also influence gene expression in vivo. Further characterisation of the DHS 1 region using CAT assays has revealed upstream activator and downstream silencer elements (Outram, 1996). Furthermore, integrity of a binding site for AP-2 was found to be essential for both these activities, again raising the possibility that differential transcription factor binding patterns at this site or other motifs within the activator and repressor regions could play an important role in regulating CD2 expression. Interestingly, AP-2 is itself inducible by retinoic acid (Zhang et al., 1996), thereby providing a link with APL and also suggesting a potential mechanism by which CD2 expression might be repressed with terminal myeloid differentiation. DHS 1 has not been finely mapped and it remains a possibility that there are a number of hypersensitive sites within the activator and silencer elements. Such phenomena could account for accessibility of the 5' flanking region of CD2 to DNase I in the context of transcriptional activation or repression.
The results presented in this thesis, not only demonstrate that the presence of DHS 1 and DHS 2 are not the sole determinants of high level CD2 expression, but are also consistent with CD2 expression being a feature of early haemopoietic progenitors with myeloid potential, in addition to T cell precursors. This hypothesis is in accordance with previous DNase I hypersensitivity assays performed in multipotential mouse bone marrow progenitor cell lines which have demonstrated that the regulatory regions of a number of genes whose products have been considered lineage restricted, including β-globin, CD38, IgH and myeloperoxidase lie within open chromatin prior to lineage commitment (Ford et al., 1992; Jiménez et al., 1992; Ford et al., 1996). Interestingly, the CD38 enhancer was found to remain accessible to DNase I following differentiation of FDCP cells into mature granulocytes (Ford et al., 1992), further supporting the theory that CD2 lies within an open chromatin configuration during normal myeloid development. Recent studies using a single cell RT-PCR approach have established that multiple lineage associated gene products are co-expressed in multipotential haemopoietic progenitors derived from human or mouse bone marrow (Cheng et al., 1996; Hu et al., 1997). Taken together these findings are more in favour of the "lineage promiscuity" model to account for inappropriate lineage marker expression in acute leukaemia; which suggests that such leukaemias originate in haemopoietic stem cell progenitors which co-express various lineage associated antigens (Greaves et al., 1986). Further support for this view is derived from the identification of rare stem cells co-expressing CD2 and myeloid associated antigens in normal marrow (Tjonnfjord et al., 1995), and from a recent large study which has correlated CD2 expression in APL, with the presence of the B lineage surface marker CD19 (Guglielmi et al., 1998).

The results presented in this thesis are consistent with a model whereby the chromatin surrounding the 5' and 3' flanking regions of CD2 is initially accessible to DNase I in CD2 positive multipotential haemopoietic progenitors, but becomes inaccessible on differentiation along B lymphoid and possibly erythroid lineages, associated with repression of CD2. Whereas, differentiation along T lymphoid and myeloid pathways is associated with maintenance of an open configuration of chromatin surrounding the gene locus. The difference in CD2 expression levels observed in mature T and myeloid cells could be accounted for by T cell specific transcription factors which activate gene expression and/or myeloid specific factors which silence CD2. In order to begin to test this hypothesis, particularly to exclude the possibility that CD2 initially lies in a closed chromatin configuration in multipotential progenitors with subsequent opening on lineage commitment, it would be interesting to perform further DNase I hypersensitivity assays in multipotential murine FDCP cells, before and after induction of differentiation.
along various pathways. These studies could be complemented by analyses to determine the presence of hypersensitive sites surrounding CD2 in multipotential progenitors and lineage committed cells isolated from human marrow by cell sorting techniques, using a PCR based approach (Yoo et al., 1996). Such analyses will be extremely important to determine whether the open chromatin configuration detected in a variety of myeloid cell lines as well as in primary APL blasts is a true reflection of the chromatin configuration of the myeloid precursors from which they arose ("lineage promiscuity" model); or alternatively indicates that chromatin remodelling at control regions of genes normally regulated in a lineage specific manner is a feature of leukaemic transformation ("lineage infidelity" model).

Nevertheless, the key question still remains unanswered as to why approximately a quarter of APL cases express detectable levels of CD2, whilst the rest do not. In this regard, it is of great interest that CD2 expression is closely correlated with hypogranular variant morphology and expression of the stem cell marker CD34; this contrasts with the classical hypergranular form of APL which typically expresses neither surface marker (Guglielmi et al., 1998). This raises the possibility that the hypogranular variant and hypergranular classical forms of APL could arise from different haemopoietic progenitor populations. Therefore presence or absence of CD2 expression in APL could be accounted for by leukaemic transformation occurring in distinct progenitors at various stages along the myeloid differentiation pathway. According to this scheme CD2+, CD34+ APL variant cases would arise from the most primitive precursors, whilst CD2-, CD34- classical cases are derived from the least primitive progenitors. If this is shown to be the case, it could account for contradictory data as to whether PML-RARα expression is detectable in multipotential progenitors (Takatsuki et al., 1993; Turhan et al., 1995). This hypothesis could be further investigated by performance of clonality studies of myeloid, erythroid, megakaryocyte and lymphoid lineages of cases of classical, and CD2 positive and negative cases of variant APL. CD2 expression in this disease could also be investigated through comparison of results of footprinting analyses around the regulatory regions of the gene in CD2 positive and negative cases of M3v, and by extension of DNase I hypersensitivity assays to include cases of classical APL.

Furthermore, it would be of interest to determine whether other epigenetic mechanisms such as DNA methylation could play a role in CD2 regulation in normal haemopoiesis and whether this is disturbed during leukaemogenesis. It is hoped that these approaches will not only establish mechanisms accounting for the detection of lymphoid markers in AML, but will also provide further insights into the processes determining expression of
lineage specific tissue antigens, the nature of the progenitors targeted by the t(15;17) and hence the pathogenesis of APL.
REFERENCES


References


References


Culligan DJ, Stevenson D, Lin Chee Y & Grimwade D (1998) Acute promyelocytic leukaemia with t(11;17)(q23;q12-21) and a good initial response to prolonged ATRA and combination chemotherapy. British Journal of Haematology, 100, 328-330.


References


References


References


References


References


References


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References


Nagpal S., Friant S., Nakshatri H. & Chambon P. (1993) RARs and RXRs: evidence for two autonomous transactivation functions (AF-1 and AF-2) and heterodimerization in vivo. EMBO Journal, 12, 2349-2360.
References


References


References


References


References


Vickers M. (1996) Estimation of the number of mutations necessary to cause chronic myeloid and acute promyelocytic leukaemias from epidemiologic data. *British Journal of Haematology, 93*, 60 (abstract suppl 1)


References


Zechel C., Shen X.Q., Chen J.Y., Chen Z.P., Chambon P. & Gronemeyer H. (1994b) The dimerization interfaces formed between the DNA binding domains of RXR, RAR and TR determine the binding specificity and polarity of the full-length receptors to direct repeats. *EMBO Journal*, 13, 1425-1433.


