The Regulation of tissue Plasminogen Activator in Rat Brain Glioma Cells

by

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A thesis submitted for the degree of Doctor of Philosophy

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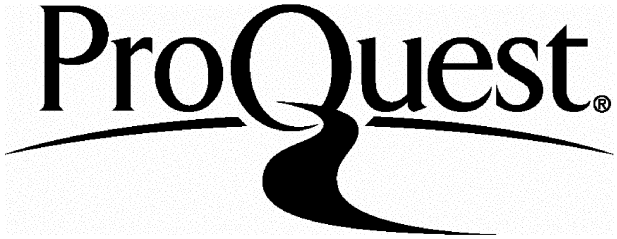

Dedicated to Dr. Joan Roscoe in her retirement.

Abstract

Plasminogen activators have been implicated in physiological events including fibrinolysis and tissue remodelling, and in invasion and metastasis by neoplastic cells.

Previous work has shown higher tissue plasminogen activator (tPA) activity in the ethylnitrosourea induced glioma line A15A5 compared with the clone from normal tissue, ARBO C9. The difference in mRNA was 10 to 20 fold while that in activity was greater, usually 50 to 80 times. The present work was directed towards finding out what mechanisms could account for these differences and for the effects of extrinsic factors on tPA activity of A15A5.

cDNA clones (approximately 1700 bases) for both lines which included the entire 3' untranslated region were sequenced. No differences were found. Both have putative AU-rich stability elements. Nuclear run-ons showed only about a 2-fold higher rate of transcription for A15A5. The transcript of A15A5 most of which is in the nucleus, shows little decay over 6 hours in the presence of actinomycin D. The level of ARBO C9 mRNA is too low to measure decay.

Many extrinsic factors affect plasminogen activator (PA) activity. These include the tissue protein plasminogen activator inhibitor-1 (PAI-1), as well as hormones and growth factors. Previous work has shown that cholera toxin (CT) increases and dexamethasone (Dex) decreases PA activity in A15A5. Present results suggest that CT primarily affect transcription of the tPA gene while the effect of Dex on PA activity is mediated through PAI-1.

The results show that post-transcriptional events are important in maintaining a high level of tPA transcripts in A15A5. The presence of most of the message in the nucleus and the lack of transcript in ARBO C9 despite a comparable transcription rate suggest regulation of stability in the nucleus.

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ABBREVIATIONS

A adenosine
AdML Adenovirus major-late promoter
ARE Adenine-Uridine rich element
Arg Arginine
AP-1 Activator protein 1
ATP Adenosine triphosphate
AU Adenine and uridine
b p Base pairs (of DNA)
C Cytidine
cAMP Cyclic adenosine monophosphate
CM Conditioned medium
CREB CAMP-response element binding protein
CT Cholera toxin
CTF/NF1 CCAAT (binding) Transcription Factor/Nuclear Factor
dATP 2'-deoxyadenosine triphosphate
dCTP 2'-deoxycytidine triphosphate
Dex Dexamethasone
DMBA 7,12-dimethylbenz(a)anthracene
DNA Deoxyribonucleic acid
DNase Deoxyribonuclease
dNTP 2'-deoxynucleotide triphosphate
DTT Dithiothreitol
ECM Extracellular matrix
EDTA Ethylenediaminetetraacetic acid, disodium salt
EGF Epidermal growth factor
ENU N-Ethyl N-nitrosourea
EtOH Ethanol
FSH Follicle stimulating hormone
G Guanosine
GTP Guanosine triphosphate
Hepes Hydroxyl piperazineethanesulphonic acid
hnRNA Heterogeneous nuclear RNA

IE Immediate early
IL 1 Interleukin 1
k b Kilobase pairs of DNA
KDa Kilodalton
LH Leutinising hormone
Met Methionine
MMP Matrix metalloprotease
mRNA Messenger RNA
Oct-1 Octamer-motif-binding protein
PA Plasminogen activator
PAI-1 Plasminogen activator inhibitor-1
PBS Phosphate Buffered Saline
RNA Ribonucleic acid
RNase Ribonuclease
SDS Sodium dodecyl sulphate
SERPINS Serine protease inhibitors
Sp1 Stimulatory protein 1
SSC Saline sodium citrate
T Thymine
TEMED N,N,N',N"-tetramethylethylene diamine
TF Transcription factor
TGF Transforming growth factor
TNF Tumour necrosis factor
tPA Tissue plasminogen activator
TPA 12-0-tetradecanoyl phorbol acetate
ts temperature sensitive
uPA Urokinase plasminogen activator
UTR Untranslated region
wt wild type

CHAPTER 1: GENERAL INTRODUCTION

1.1 Carcinogenesis

The regulation of growth and differentiation of normal cells and tissues is a precise mechanism involving both growth inhibitory and stimulatory factors. The tumour cells in contrast have the ability to elude these normal regulatory mechanisms and proliferate in an unrestrained manner.

This abnormal growth of cells or the process of tumour formation (tumorigenesis) within ordinary tissue can be detrimental to the function of normal cells. How tumours damage ordinary tissue depends on the type of tumour. Benign tumours are non-invasive and usually resemble their tissue of origin. Though they increase in size they are restricted to the site of origin and are often encapsulated and separated from the surrounding tissue. However, when fully grown the large mass can both obstruct and damage the surrounding tissue, thus interfering with the function of normal cells.

Principal characteristics of malignant tumours are that they invade the surrounding tissue and show cellular abnormalities. They also have the ability to infiltrate blood or lymphatic vessels and metastasize in to distal organs. However not all malignant tumours are metastatic. For some malignant tumours local invasion and tissue damage is sufficient to kill the host. Brain tumours being the prime example of this type of tumour.

Cancer is mainly a disease of old age. It was not a major cause

of death in the human population until the eradication of infectious diseases through improved sanitation and other advances, which lead to the rise in the average age of the population. Cancer related mortalities now account for 20% of deaths in Britain.

For victims of cancer there is a considerable improvement in prognosis if the disease is detected early and there is no invasion of vital organs. Therefore understanding the early stages of tumour development and the molecular basis for tumorigenesis would provide important information for early diagnosis and treatment of the disease.

The three major groups of carcinogens that cause tumours in mammals are carcinogenic chemicals, ionising radiation and oncogenic viruses. Investigations with chemicals have been instrumental in revealing the multi-stage nature of carcinogenesis. Some chemicals (initiators) which are genotoxic can initiate the carcinogenic process by causing alterations of the DNA sequence. Other chemicals or promoters allow the expression of the initiated phenotype, even though they cannot by themselves cause cancer.

Chemical carcinogens that can both initiate and promote tumorigenesis are termed complete carcinogens (e.g. N-ethyl N-nitrosourea), whilst those chemicals that require subsequent exposure to a promoting agent such as 12-O-tetradecanoyl phorbol-13-acetate (TPA) to produce tumours are termed incomplete carcinogens .

One of the effects of promoters is the induction of mitosis. A single dose of the tumour promoter TPA to the skins of rats, hamsters and a variety of strains of mice has been shown to induce a hyperplastic reaction within 48 hours of administration (Siskin et al., 1982). However, cell proliferation though an important event in tumour promotion is not the only effect of promoters as not all mitogenic agents acts as promoters.

There is a considerable length of time from the time of initiation to the appearance of the mature tumour, which could be up to half the life time in some instances. The events which occur during this asymptomatic latent period is the least understood of the whole process.

Due to the difficulties in studying this process *in vivo* in humans, animal models are used instead. Research on chemically induced tumours in animal models found that the pathogenesis of the induced neoplasms closely resembled tumour formation in homologous human tissue. Virtually all major forms of human cancer has been reproduced in animal species by exposure to chemical carcinogens (Farber and Cameron, 1980). Animal models also allow the induction to be carefully regulated and to follow the progression of the tumour.

Another useful technique used to study carcinogenesis is the use of tissue cultures. Transformed cells in culture provide further advantages over animal models in that they allow observation of individual populations of cells, the molecular events during initiation and progression of the tumorigenic process, and characterisation of the phenotypic changes (cellular products).

1.2 Characteristics of transformed cells.

It was the observation of transformed cells in culture in the 1960s that led to the definition of the transformed phenotype (Macpherson, 1970). These characteristics that differentiated transformed cells from the non-transformed counterparts include growth in the absence or low concentrations of serum, lack of density dependent inhibition of growth, growth in suspension or anchorage-independent growth, morphological changes and tumorigenicity on reinjection into syngeneic or immunologically deficient animals.

In contrast, normal cells in culture require high concentrations of serum which provide the growth factors required for their growth, are inhibited by cell density going into quiescence once they reach confluence and require a substratum to support their growth.

The transformation of normal cells into tumour cells can be induced in culture through infection with tumour viruses or treatment with certain carcinogenic chemicals. Cells derived from tumours induced *in vivo* also exhibit these transformed characteristics.

1.3 Fibrinolytic activity of the transformed cell.

Further studies by Unkeless et.al. (1973, 1974) on transformed cells demonstrated another distinctive feature in these cells. They found that RNA tumour virus transformed chicken embryo fibroblasts (CEF) expressed higher fibrinolytic activity than the comparable non-transformed cells. That this elevated

fibrinolytic activity was a specific cause of transformation and not simply an effect of the viral infection was demonstrated by using temperature sensitive (ts) mutants of Rous sarcoma virus (RSV). Cells infected with ts transforming mutants displayed a high fibrinolytic activity at the permissive temperature whilst cells infected at the non-permissive temperature did not. These experiments clearly demonstrated a causal relationship between transformation and fibrinolytic activity.

Others established transformation with oncogenic DNA virus can also lead to an elevation of PA activity in several cell lines. These include SV40 transformed embryonal hamster (Ossowski et al., 1973) and rat embryo cells (Pollack et al., 1974), polyoma virus transformed hamster and mouse cells, and herpes virus transformed hamster cell lines (Pearlstein et al., 1976). Furthermore, numerous cell lines of neoplastic origin including chemical carcinogen transformed early passage cultures, cell lines from experimentally induced animal tumours (Laug et al., 1975, Pearlstein et al., 1976) and cell lines derived from human tumours (Laug et al., 1975, Naggy et al., 1977) were found to posses higher fibrinolytic activity .

This higher activity has been detected in cells from non-neoplastic tissue as well. These cells were predominantly from tissue that normally undergo some type of remodelling or limited invasion (involuting mammary cells, trophoblast etc.).

Not all virus transformed cells or tumour derived cells (Markus, 1983) exhibit elevated levels of fibrinolysis. It is possible in some of these studies fibrinolytic activity may not have been

detected as plasminogen activators (PAs), the agents responsible for fibrinolytic activity can exist in different forms such as the cell associated, secreted and latent forms. The assays used to detect one form such as the cell associated form, do not detect secreted PA.

The presence of higher fibrinolytic activity in transformed cells, in cells of tumour or neoplastic origin and normal cells involved in tissue remodelling suggest fibrinolytic activity may be an inherent property of cells engaged in extracellular tissue degradation, such as that found during invasion and metastasis of tumours.

1.4 A brief history of ENU induced gliomas.

As discussed earlier certain chemicals can be used to induce tumours in animals that closely resemble homologous tumours in humans. Chemically induced brain tumours were first demonstrated by Druckrey et al (1965) who induced tumours in adult rats using N-methyl N-nitroso urea (MNU). The origins of the current study are in work which was designed to investigate the tumorigenic process in chemically induced rat brain gliomas.

In these studies the nitrosoamide ethyl nitrosourea (ENU) which is a homologue of MNU was used to induce brain tumours. ENU is a potent neural carcinogen when administered transplacentally to foetal rats. After transplacental treatment virtually all of the progeny go on to develop tumours of the nervous system. Brain tumours constitute about 60% of these, with gliomas being the majority. When BD1X rats are treated

with ENU at a concentration of 40-50 mg/kg body weight on the 15-16th day of gestation, the progeny of these rats develope cerebral tumours with an average latent period of 246 days.

1.5 The cell lines A15A5 and ARBO C9.

One of these brain gliomas was used to establish the primary culture line A15. These cells were capable of growth in a low concentration of agar (0.3%) or show anchorage independent growth. After 28 passages of A15, a positive clone from agar was established as the cell line A15A5.

Cells from the A15A5 line demonstrated several characteristics of the transformed phenotype. A15A5 cells were tumorigenic upon injection into syngeneic rats, they grew in agar and were morphologically altered compared to their normal counterparts. These cells when injected subcutaneously to young BD-IX rats produced malignant tumours in all of the recipients. The gliomas were invasive, infiltrating the surrounding muscle and tissue. The control cell line ARBO was established from the brain of a normal animal. A clone, ARBO C9 neither grew in agar nor was tumorigenic upon injection in to syngeneic animals.

1.6 Characteristics of cultures derived immediately-after (48 hours) ENU induction.

Some of the events that precede the formation of the mature glioma was investigated using cultures derived from prenatal foetal brains. Two different cultures were established (Roscoe and Claisse, 1976). A culture 48 hours after treatment with ENU (BE10) and another 48 hours after treatment with buffer (BE11).

At the time of isolation the brains from ENU treated foetuses were morphologically identical to buffer treated foetal brains.

Both cell lines were passaged in culture and their characteristics were studied. Initially cells from both cultures did not grow in agar, but after about 40 transfers cells from BE10 cultures formed small colonies several weeks after plating in agar. Also two clones of BE10 derived at the 7th and the 13th passage grew in agar and produced tumours in animals after a total of 45 transfers. The control BE11 cells neither grew in agar nor were tumorigenic after a similar number of transfers or upon further passaging. These results indicated that as early as 48 hours post-ENU treatment, cells exist that have the potential for tumorigenicity.

As it was known that other chemically transformed cells expressed high fibrinolytic activity, both tumour and prenatally derived cell lines were examined for their fibrinolytic activity using a fibrin agarose overlay assay (Hince and Roscoe, 1978). It was found that the tumour derived cell line A15A5 expressed much higher fibrinolytic activity than the comparable normal cell line.

Additionally, as BE10 cells were passaged, their transformation characteristics altered with the number of transfers. The low fibrinolytic activity of these cells at low passages (14) increased considerably by the 17th transfer. These cells were also able to survive for a long period of time (8 to 10 weeks) in agar, compared to control BE11 cells which have never survived beyond 2 weeks in agar. At this stage cells were not tumorigenic or grew in agar (Roscoe and Winslow 1980, Roscoe,

1980). After further passages (48th transfer) the cells both grew in agar and were tumorigenic in animals (figure 1.1).

This sequential change in phenotype during passage in culture could be denoted as :-

fib-, av+, ag-, tu- : fib+, av+, ag-,tu- : fib+,ag+,tu+.

(Fib = fibrinolysis; av = survival in agar; ag = growth in agar; tu = tumorigenicity).

These results from prenatal cultures show a link between fibrinolytic activity and transformation characteristics. Fibrinolytic activity and survival in agar precedes the colony formation in agar and tumorigenicity of these cells. Whether fibrinolysis is a direct cause of growth in agar is yet to be established.

Plasminogen activation was responsible for the fibrinolytic activity in these cells was ascertained through the use of Σ-amino caproic acid, an inhibitor of plasminogen activation (Alkjaersig et al., 1959). Addition of Σ-amino caproic acid to the assay medium was found to inhibit fibrinolytic activity of these cells. Furthermore omission of plasminogen from the fibrin-agarose gel assays leads to an absence of lysis zones indicating that plasminogen activation is essential for the fibrinolytic activity (Hince and Roscoe, 1978).

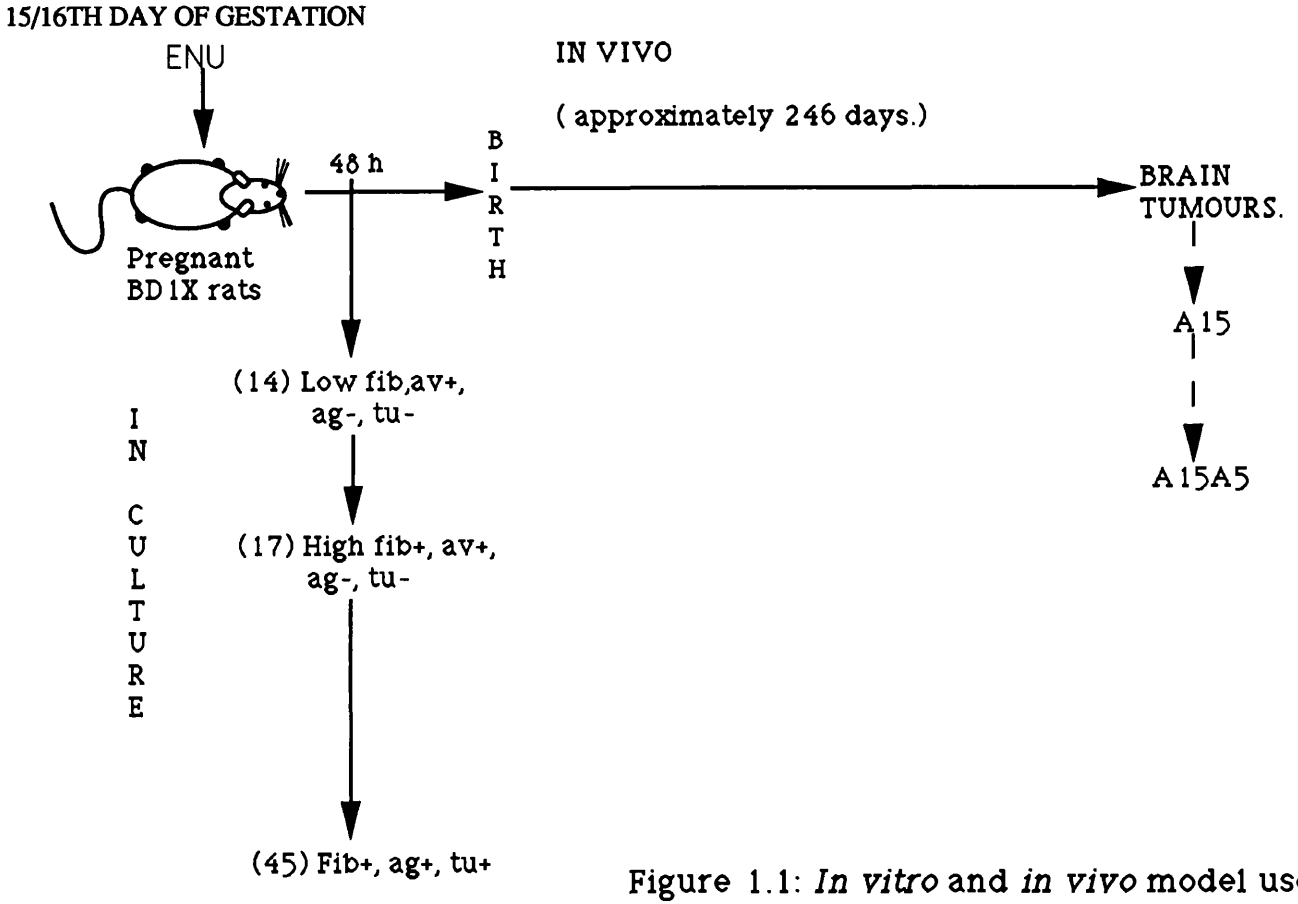


Figure 1.1: *In vitro* and *in vivo* model used to study brain carcinogenesis in the rat.*

Fib=fibrinolysis, av=survival in agar, ag=growth in agar, tu=tumorigenesis. Figures in ( ) =number of passages.

* adapted from Roscoe (1980).

1.7 The tissue-Plasminogen activator.

There are two types of mammalian plasminogen activators, the urokinase type (uPA) isolated initially from urine and the tissue type present in plasma in large quantities. The type of PA present in A15A5 cells was identified as tPA using antibody inhibition studies. Rabbit IgG antibodies to human tPA completely inhibited PA activity in the conditioned medium from A15A5 cells, whilst anti-uPA IgG had no effect on the fibrinolytic activity of these cells. Zymography studies further indicated that the molecular weight of the PA in A15A5 correspond in size to tPA (Green et al., 1986).

The human forms have been the most extensively characterised PAs. The PA protein is produced and secreted as a single chain pro-enzyme. This is converted into the 530 amino acid mature form by removal of the 35 amino acid signal and the propeptide which constitute the leader sequence.

The single chain mature tPA (sc-tPA) zymogen is cleaved between Arg_{275} and Ile_{276} to form the fully active two chain form (tc-tPA). The resulting active form consist of a N-terminal heavy chain and a C-terminal light chain, held together by a single disulphide bond (figure 1.2). The C-terminal light chain contain the catalytic activity of the protein.

The recently described rat amino acid sequence has 81% and 92% homology (Ny et al., 1988) to human and mouse tPA proteins respectively. The intron/exon boundaries of the rat tPA (Feng et al., 1990) also matches almost identically with the human tPA gene. The tPA gene consists of 13 introns and 14

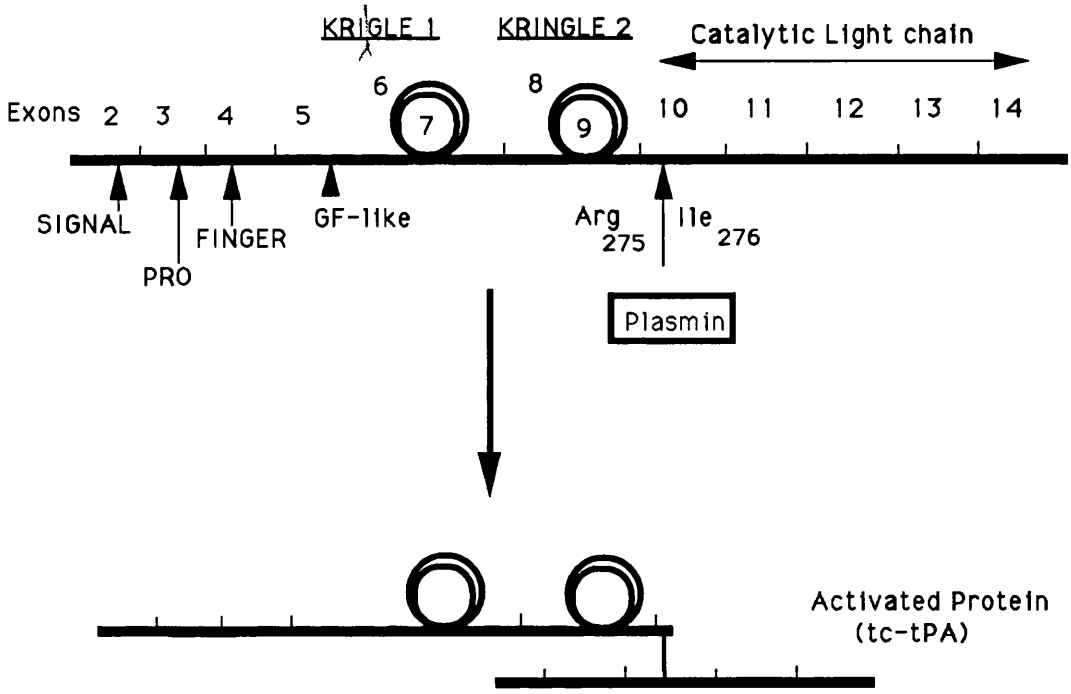


Figure 1.2: Activation of single-chain tissue Plasminogen activator to its active two-chain form (tc-tPA).
GF= GROWTH FACTOR.

exons. The 14 exons of tPA codes for several amino acid structural domains, some of which are also found in other serine proteins of the fibrinolytic system such as plasminogen, prothrombin, factor X and protein C (Pathy, 1985). Several of the structural domains have been shown to have specific functions in the protein (figure 1.3).

Exons 1 and 2 codes for the 5' leader sequence and the signal peptide of the secreted protein. The function of the 5' leader also found in other secreted serine proteases is unknown, whilst the signal peptide is thought to be involved in secretion of the protein.

Exon 3 codes for the so called pro sequence thought to be a recognition site for certain enzymes; a similar sequence is also found in serum albumin.

The finger like domain is coded for by the exon 4 and has homology to a part of the interstitial matrix component fibronectin. Finger domains are correlated with the fibrin affinity of fibronectin and are found to be involved with the fibrin binding capability of tPA.

Exon 5 codes for a growth factor (GF)-like domain. Comparable regions are found in several other proteins including uPA, clotting factor X, human and mouse epidermal growth factor (EGF), bovine prothrombin and rat FGF. These domains were termed GF-like due to their homology to certain regions of the EGF protein.

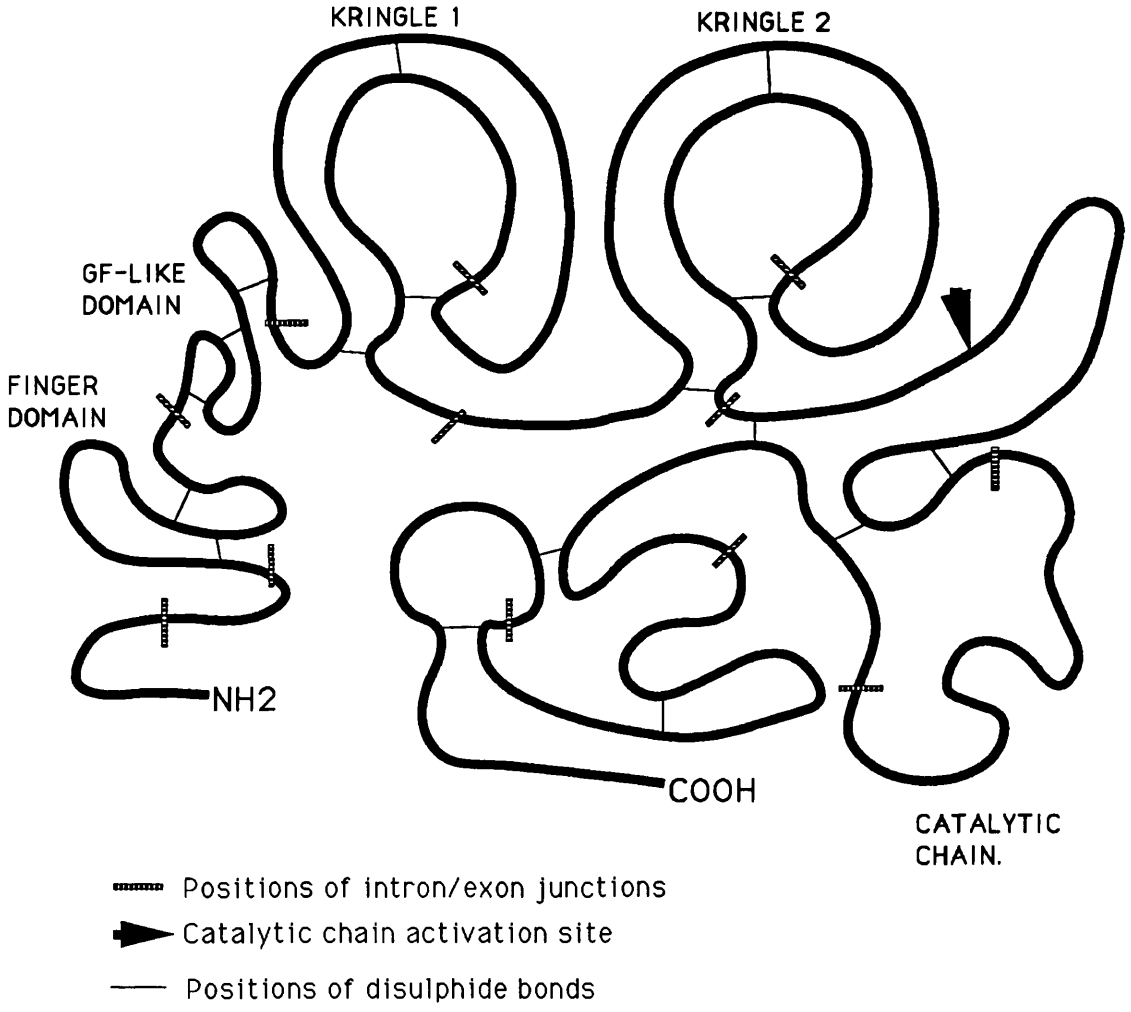


Figure 1.3 : The two dimensional amino acid structure of the tPA protein.
(adapted from Feng et al. 1990)

The GF domain of uPA has now been shown to be responsible for binding of uPA to its receptor. Apella et al. (1987) identified a 20 amino acid segment within this region of uPA that has homology to the receptor binding region of EGF. Three disulphide loops within this domain were found to confer receptor binding capacity to uPA, with the central region providing receptor specificity whilst the flanking loops providing conformation for optimal binding. The specificity of the central region may be the cause of lack of competition between EGF and uPA for the same receptor.

The amino acid sequence of tPA GF-like region also has close homology to this proposed receptor binding segment of mouse and human uPA and EGF, suggesting that the most likely function of this is in the interaction of tPA with its receptor.

The next four exons (6-9) codes for the two kringles (two exons/kringle). Kringles are triple disulphide structures found in several other proteins including prothrombin, plasminogen, uPA and hepatocyte growth factor (HGF). Kringles are thought to be important for the fibrin binding ability of some of these proteins. In tPA the majority of fibrin binding capability was shown to come from the second kringle (van Zonneveld et al., 1986) with a relatively small contribution from the finger domain. Later Bizik et al. (1993) showed that monoclonal antibodies against kringle 2 of tPA inhibit PA activity of Bowes melanoma cells considerably more than antibodies against the catalytic region. Which suggest that the kringle 2 may influence the activity of the tPA protein in ways other than its fibrin binding function. The difference in inhibition by antibodies

suggest possible protection of the catalytic region when bound to the cell surface. Incidentally lipoprotein A (LpA) the low density lipoprotein possesses 38 kringles. Of these, 24 are identical to the kringle 4 of plasminogen and lipoprotein A has been shown to compete with plasminogen for its cellular binding site (Hajjar et al., 1989). Therefore it is suggested that the affect of LpA on thrombosis may be atleast partly due to inhibition of activation of plasminogen.

In contrast to tPA, uPA has one kringle and one growth factor homologous region, and lacks the other kringle and the finger domain. These disparities reflect the differences in the properties of the two PAs. Fibrin binding is essential for tPA catalysed plasminogen activation in plasma, while uPA activity is independent of fibrin.

The last five exons (10 - 14) code for the light chain or the catalytic domain of the tPA polypeptide. This domain contains the functional site of the tPA molecule that is involved in activating plasminogen to plasmin and has homology to active site regions of trypsin, plasmin and uPA. The rat tPA active site consist of the typical catalytic triad of amino acids His_{326}, Asp_{375} and Ser_{481}.

1.8 Plasminogen activation

Plasminogen activation is the only known function of tPA and takes place in several steps. Plasminogen which is mainly produced in the liver and present in most extracellular fluids is secreted in the inactive 790 amino acid glu-plasminogen form

(92 kDa). During plasminogen activation the glu-form which has a glutamic acid residue at its amino terminus is converted to the active two chain lys-plasmin. This conversion can take place via two routes (Figure 1.4). However, during tPA mediated plasminogen activation it may be primarily directed through route B, as lys-plasminogen is activated by tPA approximately 10-20 fold more efficiently than glu-plasminogen (Hoylaerts et al., 1982). PAs cleave at Arg_{560} - Val_{561} of plasminogen to form the two chain plasmin while plasmin cleave internally to convert the glu-plasminogen to lys-plasmin. This is an autocatalytic process as plasmin can influence both latent tPA and plasminogen leading to activation of even more plasminogen.

1.9 The plasminogen activator system and extracellular proteolysis.

Plasmin is a trypsin-like serine protease and has a broad spectrum of activity. Its role encompass breakdown of fibrin clots during fibrinolysis to degradation and remodelling of the extracellular matrix (ECM). Two major matrices constitute the ECM, the interstitial matrix and the basal lamina. These matrices contain several adhesive glycoproteins which have an important role in its function. The glycoproteins affect adhesion of the cells to substrates, their migration, morphology, growth and differentiation. Two of the most important ECM glycoproteins are laminin and fibronectin

Fibronectin can specifically bind to cell surfaces and to other components of the ECM (collagen, fibrin) thus attaching cells to the structure of the ECM. Fibronectin can also acts in concert with other adhesive glycoproteins to keep normal cells in place

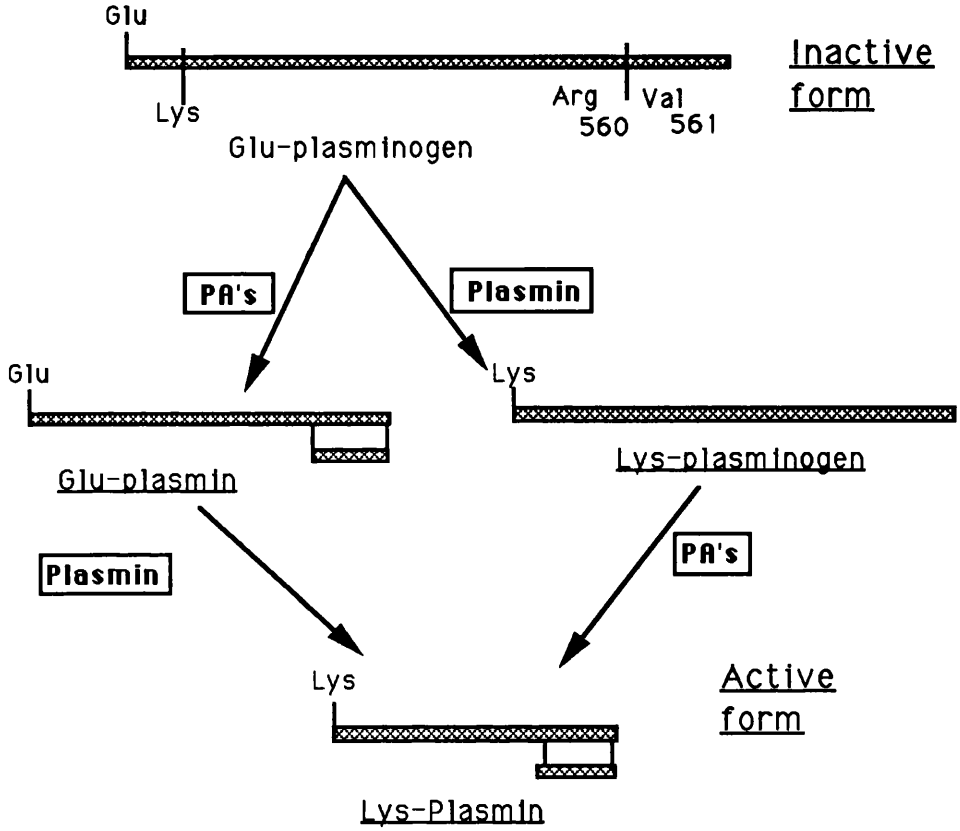


Figure 1.4 : Activation of inactive plasminogen to plasmin by plasminogen activators (PA's)

and further regulate their migration.

Laminin which is exclusive to the basal lamina has a similar function to fibronectin. It links other constituents of the basal lamina (eg. collagen type IV, heparan sulfate) to one another and epithelial cells. Laminin therefore functions in maintaining the stability of the basal lamina and in anchoring cells to the membrane.

Both fibronectin and laminin can be broken down by plasmin, and tPA is known to bind to both of these components (Salonen 1984, 1985). De Petro & Barlati (1987) demonstrated that when Chicken embryo fibroblasts are transformed with a temperature sensitive mutant of Rous sarcoma virus, they expressed a PA activator at the permissive temperature that cleaved intact fibronectin. The PA activator responsible for this activity was comparable in size (67kDa) to human tPA. These experiments showed that tPA can be activated by transformation and that it is directly responsible for degradation of fibronectin.

That tPA has the ability to cleave laminin was shown by several workers including Stack et al. (1993). These workers demonstrated that tPA activity in the highly metastatic melanoma cell line B16F10 could be induced by an 18 amino acid polypeptide (PA22-2) corresponding to part of the laminin A chain. There was also an increase in plasminogen dependent hydrolysis of laminin following PA22-2 treatment, while no increase in uPA or collagenase was found. These data suggest that tPA is the principal plasminogen activator responsible for hydrolysis of laminin by these cells. Furthermore activated

plasmin could be eluted from the surface of these cells indicating that plasminogen activation takes place on the cell surface. This peptide was previously shown to induce lung metastases by B16F10 cells.

Liotta (1986) has proposed attachment of tumours to laminin via their receptors as probably the first and most crucial step in tumour invasion and metastasis.

Another important function of plasmin is in the activation of matrix degrading metalloproteases (MMPs). Plasmin can activate two major classes of MMPs, the pro-stromelysins and pro-collagenases. Cleavage of intact fibrillar collagen is specifically limited to collagenases whilst stromelysins are active against their normal substrate proteoglycans and glycoproteins such as fibronectin and laminin as well as partially cleaved collagens.

Therefore binding to and degradation of the adhesive proteins may be an important event in invasion of metastatic tumours. Mensing et al. (1984) showed that in vitro migration of malignant cells can be enhanced by contact with fibronectin while McCarthy et al. (1986) showed that a specific regions of the human fibronectin molecule can promote haptotactic motility of melanoma cells.

Plasmin lies at the centre of a complex matrix degrading cascade. The activated plasmin has the ability to initiate the breakdown of all the major forms of ECM proteins (figure 1.5). PAs are the main regulators of plasmin levels and its aberrant expression would lead to uncontrolled destruction of tissue such

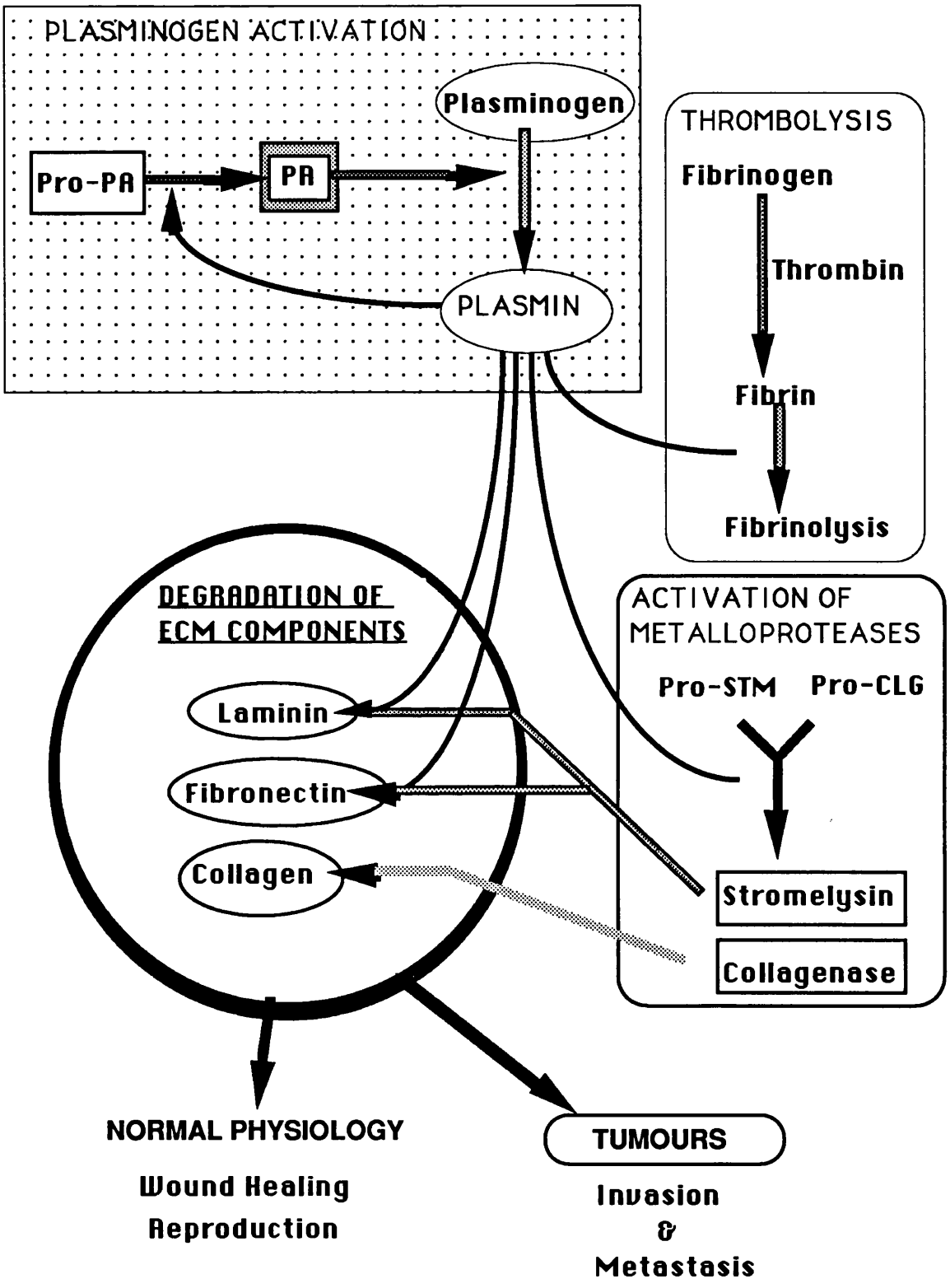


Figure 1.5 : Plasminogen activation and its relationship to other physiological events.

PA = plasminogen activator, STM= stromelysin, CLG= collagenase

as those seen during tumour invasion and metastasis.

1.10 The role of plasminogen activators in tumorigenesis.

Many tumours are known to express elevated levels of PA. There is a considerable body of evidence that indicate PAs have a role in invasion and possibly metastasis of tumours. Many of the earlier work on PA expression by tumours did not distinguish the type of PA expressed by tumour cells (Mira-y-Lopez et al.,1983,1985). The urokinase-type PA is found to be the major tumour associated plasminogen activator in non-neural tumours while several studies have demonstrated that malignant tumours of the neuroectodermal origin such as melanomas and gliomas preferentially express tPA.

Immunological studies by Bizik et al. (1990) demonstrated that several human melanoma cell lines can activate plasminogen on their cell surface and that this activity could be inhibited by monoclonal antibody to catalytic site of human tPA, but not to uPA. Other studies have also shown that melanomas express tPA (Hoal-van Helden et al., 1986).

Quax et al. (1990) analysed 22 human tumour derived cell lines and found 20 of these to express tPA. Those cell lines that expressed tPA were derived from different types of tumours including carcinomas, fibrosarcomas, a leukemia and a lymphoma indicating that tPA can be expressed by other types of tumours as well.

Other evidence for involvement of PAs in invasion has been gathered using *in vitro* invasion studies. Two main types of

models used are those composed of tissues and organs and those using reconstituted basement membranes. Work with *in vitro* models have demonstrated not only the importance of PA but also the part of PA/plasmin activated collagenases in the invasion of tumour cells. Meissauer et al. (1991) studying *in vitro* invasion by two cell lines MeWO and MelJuso, derived from two human melanomas which produced tPA and uPA respectively found antibodies against tPA and uPA could completely inhibit PA activity in their respective culture supernatants. Invasion of three different reconstituted matrices by MeWO cells was also blocked by antibodies against tPA. Later the same authors (Meissauer et al., 1992) demonstrated that both cell lines could penetrate a confluent monolayer of a non-invasive human keratinocyte cell line and this penetration could be inhibited by the removal of melanoma associated plasmin.

Mignatti et al. (1986) using a human amniotic membrane model demonstrated that anti-uPA antibodies could inhibit invasion of the amniotic membrane by human melanoma cells.

Reich et.al. (1988) also investigated the association between PAs and invasion using two malignant metastatic cell lines HT1080 and M2 which expressed higher quantities of uPA and tPA respectively. Both cell lines invaded a reconstituted basement membrane considerably more than two comparable non-metastatic cell lines ME180 and CL10 which produced much lower levels of uPA. Furthermore, synthetic inhibitors of collagenase type IV prevented invasion by the metastatic cells demonstrating collagenase type IV as the probable final protein involved in basement membrane invasion. Addition of the

plasminogen activation inhibitor Σ-amino caproic acid or antibodies against uPA and tPA also inhibited invasiveness of the malignant cells indicating that plasminogen activation is a prerequisite for collagenase production and invasion of basement membrane.

Protease mediated tissue degradation may also play a part in establishing a blood supply to or neovascularisation of established solid tumours. Neovascularisation which is especially marked around solid tumours (Folkman, 1990 and references therein) involve migration and proliferation of capillary endothelial cells and is thought to be of significance if solid tumours are to enlarge beyond few mm. Endothelial cells during angiogenesis are known to secrete plasminogen activators. Gross et al. (1983) showed that cultured bovine capillary endothelial cells produce elevated levels of PAs and collagenases when stimulated by extracts which induced angiogenesis in other cells.

These results show that elevated expression of plasminogen activators may have a crucial role in the invasion and metastasis of malignant tumours. Furthermore, they may also be involved in maintaining the viability and growth of solid tumours.

1.11 The function of the PA system in normal physiology.

The regulated expression of plasmin has a role in several normal physiological events, including dissemination of blood clots in blood vessels and after tissue injury. During wound healing the extravasated plasma or blood in the injured tissue clots rapidly to form a gel consisting of fibrin, fibronectin and platelets. This

provisional stroma is then invaded by inflammatory cells (macrophages and monocytes) followed by blood capillaries and fibroblasts. Eventually this tissue is remodelled leaving a scar composed largely of dense collagen. tPA/plasmin system is involved in several events in this process including lysis of the fibrin/fibronectin clot to fibroblast invasion and tissue restructuring (Knox et al., 1987)

Both tPA and uPA are also involved in several other tissue remodelling events directly through activation of plasmin and the secondary effects of plasmin on activation of matrix metalloproteases. In several reproductive events including spermatogenesis, mammary gland involution, ovulation and blastocyte implantation which involves tissue degradation or remodelling, there is regulated expression of one or other of the PAs (Danø et al. 1985, Saksela and Rifkin, 1988).

An important aspect of PA mediated plasminogen activation is binding of PAs and their substrates to cell surfaces which may involve specific receptors on the cell surface. A receptor for uPA has been identified (Blasi et al., 1987) and cloned (Roldan et al., 1990). Although a specific receptor for tPA has not been cloned and characterised, a 40000 Mr receptor has been recently isolated from human placental tissue that can bind both tPA and lys-plasminogen (Hajjar 1991). This protein was distinct from PAI-1, preserved tPA catalytic activity and was different from uPA binding site on the cell surface. Furthermore, Hajjar (1991) showed it to be an unique double ligand receptor on the endothelial cell surface that bind both

tPA and lys-plasminogen to generate plasmin on the cell surface. The receptor had greater affinity for tPA than for plasminogen, but there was no cross competition between the two ligands suggesting concomitant binding is a mutually favourable event for plasminogen activation.

These studies show that activation of plasminogen by its activators to plasmin is important for several physiological processes. It is probably expressed in a regulated manner at the sites of its action to prevent indiscriminate tissue damage under normal conditions, as seen with thrombolysis. The PA activity in blood is tightly regulated through balanced expression tPA and its principal tissue inhibitor PAI-1 (plasminogen activator inhibitor-1) and specific inhibitors of plasmin such as α_2-antiplasmin. In normal plasma PAI-1 is expressed at a considerable higher level than PAs to maintain a low fibrinolytic activity. Only at the site of a thrombus is there elevated PA activity. Other inhibitors of PAs include PAI-2 and protease nexin. Both of these proteins have a greater affinity to uPA than to tPA.

Unlike normal tissue, metastatic cancers express PAs in an aberrant manner which aid their growth in local sites as well as invasion into secondary site after metastasis through the circulatory system. Elevated PA activity is common in many tumours including the ENU induced rat brain glioma cells A15A5.

1.12 The expression of tPA activity in the A15A5 glioma cell line.

One of the principal events that regulate the level of a protein is the availability of its mRNA for translation. It was demonstrated (Green et al., 1986) that the total tPA mRNA level in the glioma cell line is 10 to 20 fold higher compared to the normal cell line ARBO C9, suggesting that there has been an alteration in the regulation of tPA mRNA during ENU-induced malignant transformation of the foetal brain cells.

The level of the mRNA in a cell can be regulated through transcriptional or post-transcriptional processes (figure 1.6). The rate of transcription controls the level of production of the mRNA whilst post-transcriptional events which include nuclear processing, cytoplasmic transport, translation and message degradation regulate the lifespan of the mRNA in the cell.

However, the elevated tPA mRNA level in itself does not fully account for the far greater difference (50-80 fold) in the PA activity between the cell lines A15A5 and ARBO C9. Therefore there are other differences which may be genetic or epigenetic that influence the total PA activity of these cells. Possible examples are differences at the level of translation of the tPA message or alteration in the regulation of PAI-1 the specific inhibitor of tPA activity.

This study was instigated to determine the differences in the regulation of tPA mRNA that lead to the elevated level of its

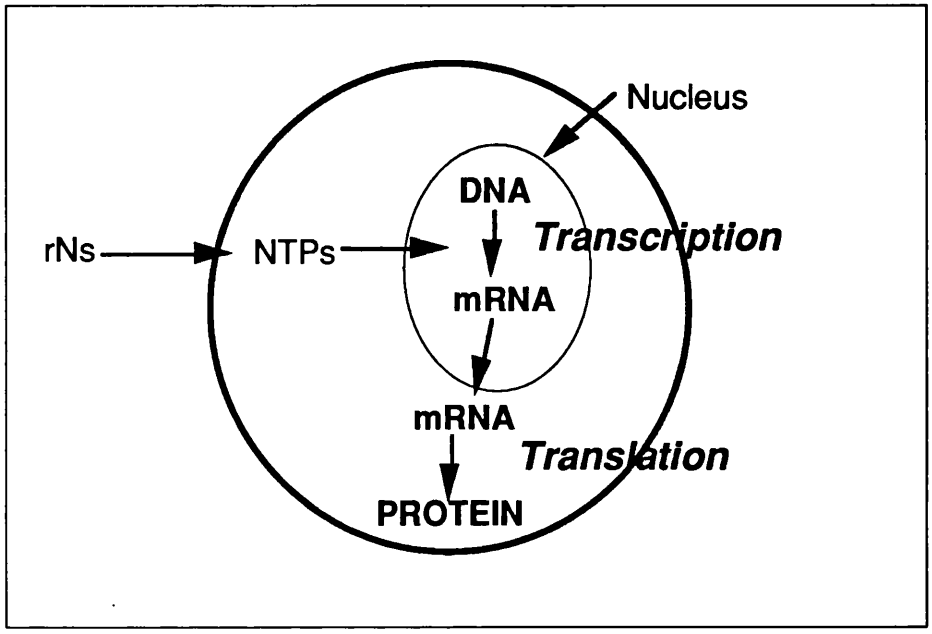


Figure 1.6 : The stages involved in regulating the expression of a protein

mRNA in the A15A5 cell line compared to ARBO C9 and also to investigate the modulation of tPA mRNA by known modulators of PA activity in A15A5.

In this project both regulation of the message at the transcriptional level (chapter 4) and stability (chapter 5) has been investigated. A cDNA sequence spanning approximately 1700 nucleotides of the tPA mRNAs including whole of the 3'UTR from the tumour and the normal brain cell lines was also determined (chapter 3). The effect of several known extrinsic modulators of PA activity were investigated for their effect on tPA mRNA (chapter 6). Finally, the expression PAI-1 the primary tissue inhibitor of tPA and its modulation in the A15A5 cell line was determined (chapter 7).

CHAPTER 2: METHODS AND MATERIALS.

2.1 Cell cultures.

The cell lines A15A5 and ARBO C9 were maintained as monolayer cultures in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 15% foetal calf serum (FCS) and grown at 37°C in a humidified atmosphere of 15% CO_2 and air. The cell lines were subcultured weekly once they reach confluence (approximately $1x10^7$ /25 cm^2 flask for A15A5 and 2 x 10^6 for ARBO C9) using a mixture of 0.05% Trypsin in a buffer with 0.43mM EDTA.

2.2 Cultures for studies in the presence of serum.

Cells were grown until subconfluent when the growth medium was removed and replaced with fresh medium. The extrinsic agents used in the study were mixed in to the fresh medium prior to its addition to the cells.

In transcription and translation inhibition studies 1μg /ml actinomycin D and 10 μg/ ml cycloheximide were use.
Cholera Toxin (CT) was used at a concentration of 10ng /ml and dexamethasone at 10^{-7} M (final concentration).
For the control cultures during actinomycin D studies an appropriate volume of acetone, which is used to prepare the stock actinomycin D solution was added to the cells; for studies

with dexamethasone, ethanol (in which the stocks of the hormone is prepared) was added to the controls.

2.3 Cell cultures in the absence of serum.

After the cells have grown to subconfluence, the growth medium was removed and the culture dishes were washed 2-times with DMEM to remove FCS. Fresh medium (DMEM) lacking serum was the added to the cultures and the cells were maintained at 37°.

2.4 DNA Sequencing

The sequencing of single-stranded M13 clones were performed using the 'Sequenase^{TM} version 2.0' kit supplied by the United States Biochemical Corporation. The procedure uses a modification of the chain termination method of DNA sequencing (Sanger et al., 1977) and utilizes the enzyme 'Sequenase', a modified form of T7 DNA polymerase. The labelled products were heated for 80°C for 2 min before running on polyacrylamide denaturing gels.

2.5 Sequencing gel electrophoresis.

Electrophoresis of sequencing products were carried out in (7%) polyacrylamide / 8M urea denaturing gels. The gels were pre-warmed for 10 min at 35W and run in 1x TBE at 30W. For extended sequencing electrolyte buffer gradients (Sheen and Seed, 1988) were used instead of wedge gels. The electrolyte gradient is achieved by initially running the gels with 0.5x TBE in the upper chamber and 1x TBE in the lower chamber. After

few hours the lower chamber is made up to 1M sodium acetate, which creates a electrolyte gradient causing the upper part of the gel to be hotter than the lower part. After electrophoresis the gels were fixed in 12% (v/v) methanol and 10% (v/v) acetic acid for 15 min, dried under vacuum at 80°C and exposed to X-ray film.

2.6 Plasmid preparations.

2.6.1 Small scale plasmid preparations.

Alkaline lysis procedure was used (Sambrook et al., 1989): 5ml of LB medium containing the appropriate antibiotic was inoculated with a loopful of bacteria from a frozen stock and incubated overnight at 37°C, shaking at 250 rpm. 1.5 ml of the culture medium was centrifuged and the pellet resuspended in 100µl of ice cold solution of 50mM glucose, 25mM Tris-Cl and 10mM EDTA and left for 5 min at room temperature. A 200µl freshly prepared solution of 0.2M NaOH and 1%SDS was added, mixed by inverting several times and left on ice for 5 min. 100µl of an ice cold solution of 3M/5M potassium/acetate was added and the tube vortexed for 10 sec. The tube was incubated for 5 min on ice, centrifuged at 4°C for 5 min and the supernatant removed to a new tube. The protein was removed using an equal volume of phenol : chloroform (50:50) and the plasmid DNA precipitated with 2 volumes of 100% ethanol. The pellet was washed with 1ml 70% ethanol, dried at room temperature for 5 min and resuspended in 20µl TE pH8.0

2.6.2 Large scale plasmid DNA preparations were carried out using the Qiagen plasmid Kit supplied by Qiagen Inc.

2.6.3 Non-denaturing agarose gels

DNA fragments that were "restricted" were analysed by horizontal agarose 'mini-gels' (BRL apparatus). The agarose concentrations varied between 0.6% and 1.5% (w/v) depending upon the size of the DNA fragment of interest. The gels were made and run as described by Sambrook et al., (1989) in TBE electrophoresis buffer.

2.6.4 Isolation of DNA fragments

DNA fragments for use in slot blots and for probing Northern blots were cleaved from their vectors, size separated in low-melting point (LMP) agarose gels and purified from the gels by the following method. This method employs Promega's "Magic clean-up" columns, but the solubilization of agarose prior to addition to the columns is adapted from a different protocol (original source unknown).

The minimum gel size containing the required fragment was excised from the gel and weighed to determine the size. The gel was melted by adding 500µl of buffer A (0.75M NaCl, 50mM MOPS pH7.0, 15% EtOH) per 100mg of gel and incubated at 65° for 10min. Mixed well by vortexing, rapidly added to a tube containing urea (DNase,RNase free) at a concentration of 200mg / 100mg of gel and mixed by inverting until the urea is completely dissolved (urea preserves the agarose in liquid form even at low temperatures). 500µl of agarose solution was

mixed with 1ml "Magic clean-up" resin and DNA isolated as in the Promega protocol.

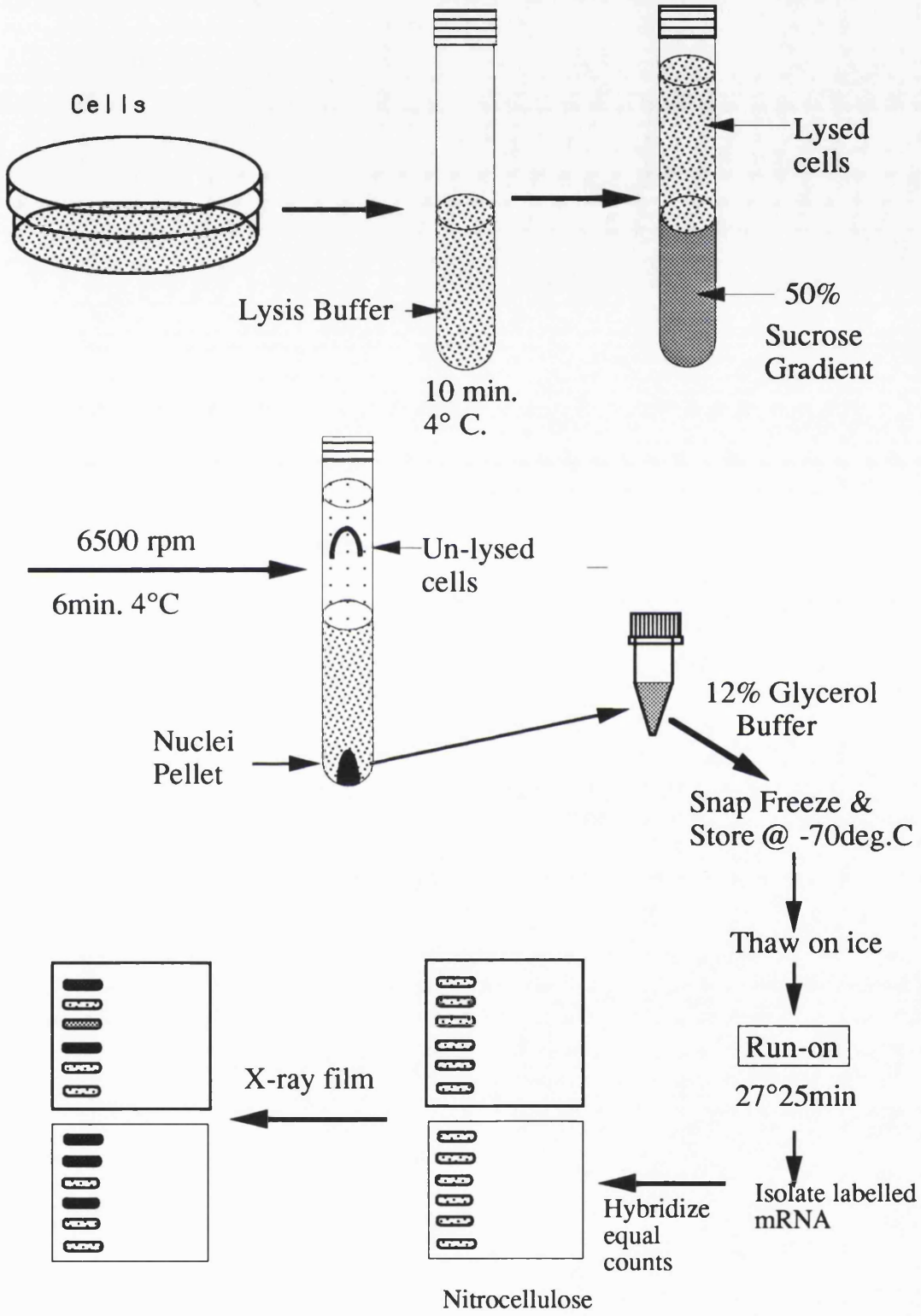
2.7 The Nuclear Run-On Transcription Assay.

All ingredients used in transcription assays were ultrapure and, RNase and DNase free. All solutions were made in DEPC treated SDW.

2.7.1 Isolation of nuclei.

The initial work was carried our using a procedure based on the method of Greenberg and Ziff (1984). Detectable transcription could not be achieved from this method, therefore the method was extensively modified as discussed in chapter 4. For each assay, nuclei were isolated from approximately 2.5 x 10^7 - 1 X 10^8 semi-confluently growing cells. The cells were scraped into PBS using a "silicon" rubber policemen and spun at 3500 rpm for 4 mins in a Sorval SS45 rotor. The supernatant was removed, the cell pellet resuspended in 4 ml lysis buffer (0.75% Nonidet P-40, 10mM Hepes, 10 mM NaCl, and 3mM $MgCl_2$) and left on ice for 5 mins. The cells were resuspended again and the 50% sucrose gradient buffer(0.2% Nonidet P-40, 10mM Hepes, 10 mM NaCl, and 3mM $MgCl_2$) added to the bottom of the tube (figure 2.1). The gradient was centrifuged at 6500 rpm for 6 mins, supernatant was removed leaving the nuclei on the bottom and any unlysed nuclei attached to the side of the tube. The lysis and the sucrose gradient separation was repeated and the nuclei pellet resuspended in the 12% glycerol buffer (12% glycerol, 5mM Hepes, 0 .1 mM EDTA, and 5mM $MgCl_2$), snap frozen in liquid nitrogen and stored at -70

Figure 2.1 : The nuclear run-on transcription assay.



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2.7.2 Transcription reaction

RNA transcription in the isolated nuclei was carried out in a transcription buffer composed of 50mM Hepes pH7.9, 120mM KCl, 5mM DTT, 5mM Magnesium acetate, 2mM Manganese chloride, 0.4 mM EDTA, 0.3 % (v/v) Tween 80, 28mM Creatine Phosphate, 300μg/ml Creatine Kinase, 4mM each of ATP, GTP and UTP; 0.1mM CTP, 25μg/ml BSA, 400U/ml RNAguard and (^{32}P)CTP (250 uCi/assay).

The reaction was allowed to proceed for 20 to 25 min at 28° C. The radiolabelled newly synthesized RNA was isolated according to Celano et al. (1989) which is an adaptation of the method of RNA isolation of Chomczynski and Sacchi (1987). The isolated RNA was washed 1x in 75% EtOH, 1x in 100% EtOH and the pellet dissolved in 200μl TES. 2μl of RNA in solution was added to 5ml of UniScint BD scintillant and the specific activity counted in a LKB 1211 RackBeta counter. Equal total amounts of radioactivity (labeled RNA) from each reaction were hybridized to separate nitrocellulose membrane strips.

2.7.3 Slot Blot Analysis

In nuclear run-on assays the relative concentrations of specific mRNAs were analyzed by slot blot analysis by hybridizing labelled RNA with nitrocellulose membrane (Schleicher and Schuell, 0.45μm) bound DNA (2 or 4μg DNA/slot). Prior to its application to the slot blot the dsDNA was denatured by boiling in 0.25M NaOH, chilled and neutralised with 1M ammonium

acetate. The blots were pre-hybridized at 42°C for 6-8 hours before addition of the labelled mRNA.

The amount of incorporated radioactivity was determined by scintillation counting and equal amounts of label were added to the blots. After hybridizing for 72 - 80 hours the blot were washed sequentially as follows to remove non-specifically bound label.

2 x SSC, 0.2% SDS RT 15 mins. 2-times

2 x SSC, 0.2% SDS 55° 15 mins.

2 x SSC, 0.1% SDS RT 15 mins.

Pre - hybridization solution:

50% formamide, 50 mM sodium phosphate (pH6.5), 5 X SSC, 5 X Denhardts, 0.5% SDS, 10 X dextran sulphate and 25 µg/ml denatured herring sperm DNA.

The hybridization solution was above (fresh) and the labelled mRNA.

2.8.1 Determination of the level of transcription inhibition by actinomycin D - by incorporation of ^{3}H uridine.

[The method was adapted from Ascoli et al., (1983)].

To 1x 10^{5} cells growing in 3.5 cm tissue culture plates, ^{3}H uridine (1μCi/ml) and actinomycin D (1μg/ml) were added and incubated for the required time interval. For the control cultures during actinomycin D studies an appropriate volume of acetone, which is used to prepare the stock actinomycin D solution was added. Two plates per time point was used. At the end of incubation, dishes were placed on ice and washed 5 x with 2ml ice-cold PBS. After adding 2ml cold TCA at 10% w/v the plates were left on ice for 30min. The TCA was removed and further washed 2x with 10% TCA. The cells were solubilised with 1 ml NaOH (0.5N) and the level of incorporation of ^{3}H uridine to the message was determined through scintillation counting (LKB 1211 RackBeta counter) using UniScint BD as a scintillant.

2.8.2 Determination of incorporation of ^{35}S methionine after inhibition of translation by cycloheximide.

Exponentially growing cells at a similar concentration to that in the ^{3}H uridine incorporation assay was used. The growth medium was aspirated and the plates were washed twice with methionine-free (Met-) DMEM to remove traces of serum left. 2ml of fresh (Met-) DMEM containing ^{35}S Met (2μCi/ml) and cycloheximide at 10μg/ml were added and the plates incubated for the required time period. Subsequently the plates were washed 5x with PBS, the protein precipitated by 10% (w/v) TCA and the level of ^{35}S methionine in whole cell lysates determined by liquid scintillation counting.

2.9 RNA Methodology

2.9.1 RNA Isolation

Total cellular RNA was isolated by a modification of the method of Chomczynski and Sacchi (1987). Monolayer cultures were lysed on the culture plate by the addition of solution D (4 M guanidinium thiocyanate, 25mM sodium citrate, O.5% sarcosyl, and 0.1M 2-mercaptoethanol) directly on to the plate. The lysates were transferred to microfuge tubes and either used immediately or stored at -70° C for extraction of RNA. For isolation of RNA, sodium acetate (0.3 M) was added and proteins were extracted into water-equilibrated phenol and chloroform-isoamyl alcohol (49:1). RNA was precipitated with isopropanol, and the pellet was washed with ethanol, dried, and resuspended in DEPC-treated sterile water. RNA concentration was determined by absorbance at 260 nm.

2.9.2 Northern blotting and hybridization

RNA gel electrophoresis was performed in 1.1% denaturing agarose slab gels in 1x gel running buffer (5x buffer = 200mM morpholinopropanesulphonic acid (MOPS) (pH7), 50mM sodium acetate and 5mM EDTA pH8.0 prepared in water) and 2.2M formaldehyde. The agarose was melted in SDW and cooled to below 60°C prior to addition of gel running buffer and formaldehyde. The samples (approximately 10µg RNA) were prepared in 0.5x running buffer, 50% (v/v) formamide and 5% (v/v) formaldehyde and were incubated at 60°C for 10 minutes. Each sample was mixed with 3µl of loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene

cyanol) and 1µl ethidium bromide (1mg/ml) before loading into the wells. The gels were run in 1x running buffer at 65V for 4 to 5 hours. After electrophoresis the gels were washed according to Sambrook et al.(1989). That is, rinsed in sterile distilled water (SDW) twice, soaked in 0.01N NaOH for 15 min, rinsed in SDW and washed in 20x SSC for 40 min.

Prior to transfer of RNA the nitrocellulose (NC) membrane was wetted in SDW for 5 min and soaked in 20x SSC for 10 min. The RNA was transferred overnight in 20x SSC and the membrane was baked at 80° for 2 hours to fix the RNA. Northern hybridization was performed using radiolabelled DNA fragments; before hybridization the NC membranes were pre-hybridized for 4 to 6 hours.

2.10 Radiolabelling of restriction fragments

Double-stranded, linear DNA fragments were radiolabelled according to Feinberg and Vogelstein (1983) using the random primer labelling kit supplied by the Boehringer Mannheim company. Approximately 20 ng of DNA was denatured by boiling for 7 to 8 minutes, quenched on ice and labelled with (^{32}P) dCTP using 1 unit of Klenow fragment. The labelled probe was made up to 100µl with 1x TE (pH8.0) and the unincorporated nucleotides were removed by centrifugation through a Sephadex G50 spun-column as described by Sambrook et al. (1989). The probe was boiled for 10 min and quenched on ice prior to addition to the hybridization buffer.

The hybridization was carried out overnight at 42°C. The blots were washed and exposed to X-ray film at -70°C. The pre-hybridization and hybridization solutions, and conditions for washing of the blots are as in section 2.7.3

For reprobing the blots were stripped according to Sambrook et al. (1989).

2.11 Standardization of signals.

Intensity of the signals were determined using a Joyce Lobel densitometer. Signals from run-on assays were standardised against rat repetitive (4D12) sequences (Witney and Furano, 1984), whilst signals from northern blot analyses were standardised against ß-actin mRNA levels.

2.12 Other solutions and buffers.

6 x agarose gel loading buffer (1.25%(w/v) bromophenol blue, 30% (v/v) glycerol)

7% Polyacrylamide gels ($50ml^{-1}$) = 21g urea, 8.75ml of 40% acrylamide (38% acrylamide, 2% bis-acrylamide), 10ml TBE (5x).

Denhardt's Solution: (100X stock, L^{-1}) 20g ficoll, 20g polyvinyl pyrrolidone, 20g BSA.

L Broth (LB),(L^{-1}) 10g tryptone, 5g yeast extract and 10g NaCl; pH adjusted to 7.0 with 10M NaOH.

PBS (phosphate-buffered saline); (L^{-1}) 6.1g NaCl, 0.135 g KCl, 0.96g $Na_2HPO_4.2H_2O$, 0.17g KH_2PO_4. pH was adjusted to 7.4 using HCl.

SSC (saline sodium citrate): (20x) : 3M NaCl and 0.3M tri-sodium citrate. pH was adjusted to 7.0 with NaOH.

TAE (tris-acetate-EDTA): 40 mM tris-Cl, 20 mM Na acetate, 2mM EDTA. pH was adjusted to 8.3 using NaOH.

TBE (tris-borate-EDTA): 100mM Tris-HCl, 100mM boric acid, 2mM EDTA pH8.35

TE (Tris-Cl/EDTA): 10 mM tris-Cl, pH 7.5, 1mM EDTA.

Tris-Cl: 1 M tris (121.14g L^{-1}) was prepared and the pH was adjusted appropriately with HCl.

2.13 Materials

All Restriction Enzymes, Low-melting point agar and urea - Gibco/BRL

Acrylamide and Bis-acrylamide- BDH chemicals

Bacto-tryptone and Yeast Extract- Difco Labs.

Creatine Phosphate, Creatine Kinase, "Magic clean-up" colums - Promega

Electrophoresis apparatus- BRL

Films- Kodak X-Omat AR and XAR-5 ; Fuji 100 RX; Polaroid 667

General laboratory plasticware - Marathon Laboratory Supplies.

Hepes (RNase, DNase free), KCl, DTT, Magnesium acetate, Manganese chloride (RNase, DNase free), EDTA (RNase, DNase free), Tween 80, Ammonium acetate (RNase, DNase free), Nonidet P40, DEPC, Trizma base (RNase, DNase free), Glycerol (RNase, DNase free), Urea (RNase, DNase free), Actinomycin D, Cycloheximide- Sigma chemical company.

Nitrocellulose membrane (BA 85) - Schleicher and Schuell

Nucleotide triposphates, BSA, RNAguard, DNase 1 - Pharmacia.

Qiagen Plasmid preparation kit- Qiagen Inc., Hybaid.

Random primer Labelling kit - Boehringer Mannheim.

Sequenase (Version 2) kit - United States Biochemicals, Cambridge BioScience.

UniScint BD - National Diagnostics, U.K.

CHAPTER 3. THE cDNA SEQUENCE OF tPA FROM A15A5 AND ARBO C9.

3.1 Introduction

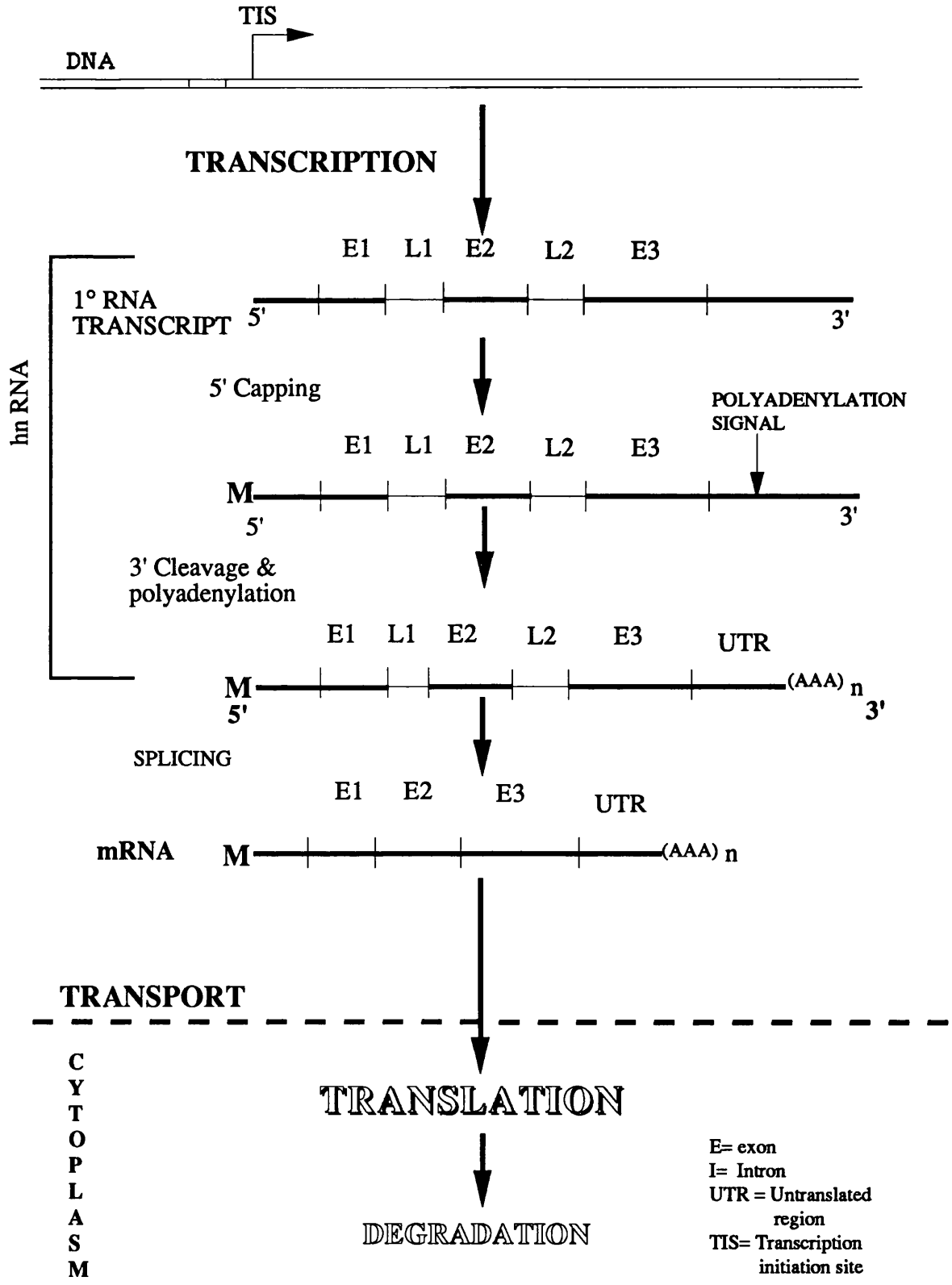
As discussed in the introduction the tPA message level in the malignant tumour cell line A15A5 is approximately 10-20 fold greater compared to its normal counterpart ARBO C9, while the difference in PA activity was even greater (60-80 fold).

The higher level of the message in a cell could be due to regulation at the level of transcription or during many of the post-transcriptional processes. Post-transcriptional events include 5' end capping, splicing, 3' polyadenylation, transport of the mature message to the cytoplasm, translation and finally degradation of the message (figure 3.1).

Much of the early work on the cellular regulatory events that determine the message level concentrated primarily on mRNA transcription and the 5' promoters that regulate transcription (Chapter 4 for details.).

This bias towards the 5' end of the molecule meant little attention was paid to the 3' UTR of the mRNA. Attention was probably further diverted away from the 3'UTR as having any significant role in message regulation due to its absence in prokaryotes. Therefore the 3'UTR was treated as a filler between the coding region and the poly-A tail containing

Figure 3.1 : The pathway of nuclear processing of newly synthesized RNA.



nothing more than the signal for polyadenylation. However, especially within the last 5 years a considerable amount of evidence has been gathered by different workers to suggest that the 3'UTR has an important role in mRNA regulation.

3.2 The role of the poly-A tail in mRNA regulation.

Several functions attributed to sequences in the 3'UTR include message localisation, polyadenylation, stability, translation and even regulating other mRNAs through a trans-acting mechanism.

The first of these important signals identified was the transcription termination and the polyadenylation signal AAUAAA, generally found 20-30 nucleotides upstream from the poly-A tail. This sequence and a GU-rich sequence found immediately beyond the site of polyadenylation are the minimal sequences required for polyadenylation (Levitt, 1989). Base substitutions within the AAUAAA signal has been shown to both reduce the efficiency of cleavage and in turn affect the level of polyadenylation (Sheets et al., 1990). Two protein factors, the poly-A polymerase and the cleavage and polyadenylation factor (CstF), that bind to the AAUAAA and are essential for cleavage and polyadenylation has been purified (Takagaki et al., 1990).

The poly-A tail of eukaryotes can vary from 60-200 nucleotides. There is conflicting evidence whether the poly-A tail has a role in the function of mRNA. Some workers have

suggested that it has no real input on message behaviour, while majority of the evidence suggest that it can affect several post-transcriptional events.

One of the roles ascribed to the poly-A tail has been activating latent mRNAs. The expression of tPA during oogenesis and oocyte maturation is a paradigm for this process. During oocyte maturation little or no mRNA transcription takes place. Many of the proteins, including tPA, translated during this period come from dormant mRNAs stored in the cytoplasm. These mRNAs are originally transcribed during the earlier oocyte developement stage. The dormant mRNAs have very short poly-A tails compared to their homologues in somatic cells. In the primary mouse oocyte the dormant tPA message has a poly-A tail of 30-40 nucleotides (Huarte et al., 1987). The stored mRNA is translationally activated during meiotic maturation by readenylation of its poly-A tail in the cytoplasm.

The dormant short tailed tPA mRNA is not a product of nuclear events, but a product of cytoplasmic processing. The nuclear tPA message is a 300-400 poly-A nucleotide product which is deadenylated in the cytoplasm (Huarte et al., 1992). Therefore in contrast to somatic cells deadenylation during oogenesis does not lead to the destruction of the message .

3.3 Involvement of 3' UTR cis-elements in mRNA processing.

Readenylation of the dormant tPA during translation activation was found to require two sequence elements in the 3'UTR. One is the AAUAAA polyadenylation signal. When a tPA transcript with a short poly-A containing the mutated form of the signal AAGAAA in the 3'UTR was injected into primary oocytes the message remains deadenylated as the wild type. However, when the oocytes were allowed to mature the mutated transcripts undergo further deadenylation while the wild type transcripts remain in an adenylated state.

The other element required for readenylation was an element rich in A and U nucleotides termed the CPE (cytoplasmic poly-A element). The CPE was initially determined in Xenopus oocytes and was found to contain the sequence UUUUUAU (Fox et al., 1989). The mouse and the rat tPA messages do not contain an exact copy of this sequence, but has the closely related AUUUUAAU motif within a region high in AU nucleotides. Removal of this AU rich region from tPA leads to neither deadenylation nor readenylation of injected mutant transcripts. Which suggest while adenylation require the AAUAAA signal, the AU rich element is required for both deadenylation and readenylation of the message.

In somatic cells the AU-rich sequences in the 3'UTR are thought to be involved in message stability, especially regions containing repeats of the AUUUA pentamer. Studies on chimeras between human c-fos and ß-globin showed that

3'UTR's had the most dramatic effect on the stability of these genes (Kabnick and Housman, 1988). Both c-fos and ß-globin have AUUUA sequences in the 3'UTR, with c-fos having 3 AUUUA's and ß-globin 1. Deletion of the AU-rich element in c-fos lead to slowing of the poly-A shortening rate and an increase in its stability.

Bosma and Kooistra (1991) found the two forms of human PAI-1 mRNA in Hep G2 cells to have differential stabilities. Although both forms could be induced by phorbol esters, the larger form containing two AUUUA pentamers was degraded much more rapidly than the minor form.

Secondary structures such as stem-loops and pseudoknots adopted by mRNA are also important determinants of mRNA degradation. The iron response element (IRE) is the most characterised secondary structure. IRE sequences are inverted repeat sequences that can fold into stem-loop structures (Casey et al., 1989). They are found in both the 3'UTR of the transferrin receptor and the 5'UTR of the ferritin gene. These two genes are involved in importing iron into the cell and binding to intracellular iron respectively, therefore they regulate the concentration of cellular iron levels. When the intracellular iron levels are low IRE-binding proteins (IRE-BP) binds to the IREs causing two effects. It inhibits the translation of ferritin mRNA and simultaneously suppresses the degradation of transferrin receptor mRNA therefore increasing the level of iron available to the cell.

In prokaryotes stem-loops has been shown to effectively impede processive activities of 3'-5' exonucleases (McLaren et al., 1991), while the cleavage targets for RNaseIII also appear to involve both secondary structures as well as primary sequences (Krinke and Wulff, 1990).

Recently evidence have been put forward by Rastinejad and Blau (1993) that 3'UTRs of mRNA can behave as trans-acting factors to regulate other messages in the cell. This process was demonstrated for three differentiating muscle specific genes. During myogenesis, simultaneous growth and differentiation are antagonistic to each other. That is, muscle cell that are undergoing differentiation do not multiply whilst those that are growing do not express their cell specific genes. Furthermore, addition of growth factors to differentiating cells leads to suppression of differentiation, and arrest of cell growth in dividing populations causes the cells to undergo differentiation. These workers found 3'UTRs from three specific muscle genes troponin, tropomyosin and α-cardiac actin can suppress proliferation of non-muscle 10T1/2 fibroblasts whilst augmenting differentiation of wild type muscle cells. These data suggest that these 3'UTRs can act not only within myogenic cells, but also in non-homologous cells as trans-acting regulators of other genes.

The regulatory sequences in the 3'UTR involved in controlling

mRNA processing and message stability are proposed to act in conjunction with trans-acting factors as several proteins have been characterised with the ability to bind to the 3' UTR of mRNA. These factors can be put in to several categories including AU-rich element (ARE) binding proteins, several of which ranging in size from 20 - 70 kDa have now been characterized (Bohjanen et al., 1991b), poly-A binding proteins (PAB) (Sachs and Davis, 1989), which binds to poly-A tracts, and in yeast co-operate with poly-A-nuclease (PAN) to degrade poly A tracts (Sachs and Deardoff, 1992) and the poly-A polymerase and CstF discussed earlier.

3.4 The effect of mutations in the coding region on mRNA stability.

Another way that sequence alterations could affect tPA gene expression is mutations in the coding region of gene. Frameshift mutations or nonsense mutations within the coding region generally leads to premature translation termination. Several reports have shown that premature translation termination leads to rapid degradation of the mRNA and thus lowering of the message levels. Bauman et al. (1985) showed frameshift mutations within the mouse immunoglobin *m u* gene leads to abnormally low level of its message. The position of the mutation was important; as the distance from the translation initiation site increased, there was a progressive increase in the level of the message; that is the further the translation was allowed to proceed the more stable was the message. However, this relationship was not strictly

proportional, mutations in the first half of the coding region caused considerably lower mRNA levels than those in the second half of the coding region.

Daar and Maquat (1988) found nonsense codon mutations within the coding region of the mRNA that disrupts the human anaemia inducing gene triosephosphate isomerase lead to a 20% reduction in the half life of the mRNA compared to its normal levels. Unlike the *mu* gene the level of the message was not affected by the position of the mutation relative to the initiation codon. All mutations caused a similar level in reduction of the message.

Mutations within the coding region could also affect the structure of the protein. The mature tPA gene contains a number of structural domains that are homologous to domains found in other serine proteins. Evidence so far suggests that each domain of the protein contributes to some aspect of its activity. For example, the EGF-like domain is probably involved in receptor binding, Kringle 2 with fibrin binding and the light chain providing the catalytic activity. *In vitro* studies have shown that alterations of the amino acid sequence in individual domains would abolish their functions (Bennett et al., 1991). Apella et.al (1987) showed that modification of the receptor binding site of uPA would inhibit the receptor binding capacity of the protein, while others have demonstrated changes in the catalytic domain would influence the activity of the protein.

Therefore mutations within the coding region and the 3'UTR could effect the tPA transcript in several ways including its mRNA stability and its translation. Therefore it was important to sequence the tPA mRNA from the glioma cell line and its normal homologue ARBO C9 to determine whether differences in the sequence could account for some of the differences in the mRNA level and the activity between the cell lines. Furthermore when this project was initiated the mRNA sequence of the rat tPA gene was unknown, although the cDNA for human tPA has already been cloned and published (Pennica et al. 1983). Since then both the cDNA (Ny et al., 1988) and the genomic sequences (Feng et al, 1990) of rat tPA have been determined.

3.5 RESULTS

Two cDNA fragments of the rat tissue plasminogen activator gene was sequenced (figure 3.2). A 1691 nucleotide fragment from the ENU induced tumour cell line A15A5 and a 1697 nucleotide fragment from the control cell line ARBO C9 (Appendix A) were sequenced[1].

The difference in the length was due to an additional 6 bases in the poly-A tail of the ARBO C9 fragment and not due to any differences in the coding or the 3'UTR of the two sequences.

These fragments correspond to 811-2502 nucleotides (figure 3.3) of the published sequence (Feng et al, 1990). The coding region of this sequence (956 bases) covers part of exon 7 or part of kringle 1, kringle 2 and whole of the C-terminal active site. The 3'UTR of both sequences were 735 bases in length and correspond to 1767-2499 nucleotides of the published sequence. The poly-A addition signal AAUAAA lies 28 - 33 bases upstream of the poly-A addition site (Appendix A).

Although it was thought that there may be differences between the two fragments that could account for the altered expression of tPA protein and its mRNA in A15A5 cells, no differences could be found within the untranslated region or in the coding region between the two sequences from A15A5 and ARBO C9.

[1]The preparation of the cDNA library and the cloning of the fragments in to the pUC vectors were carried out by Linda J. Green, a previous member of this laboratory.

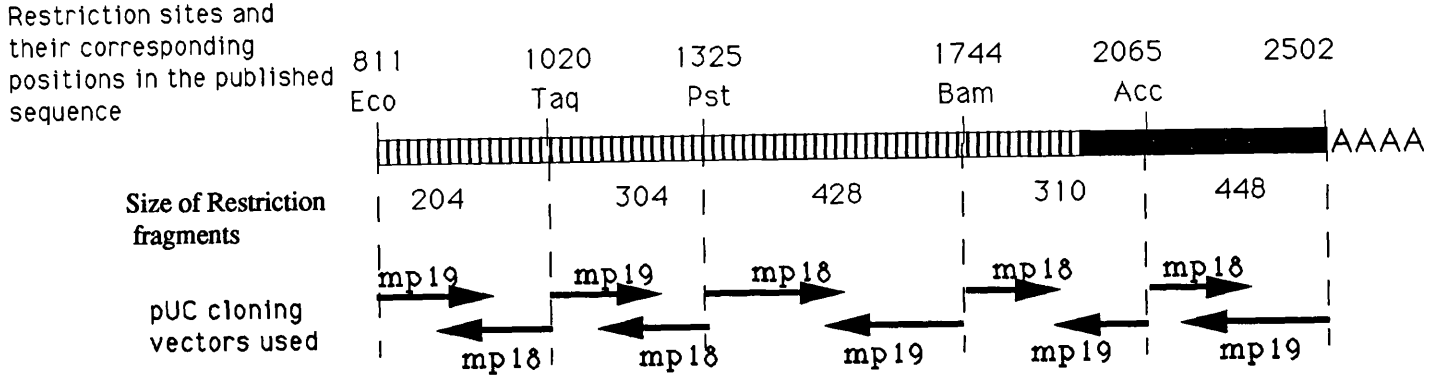


Figure 3.2 : The length of the tPA cDNA fragments sequenced, the restriction sites and the pUC cloning vectors used.

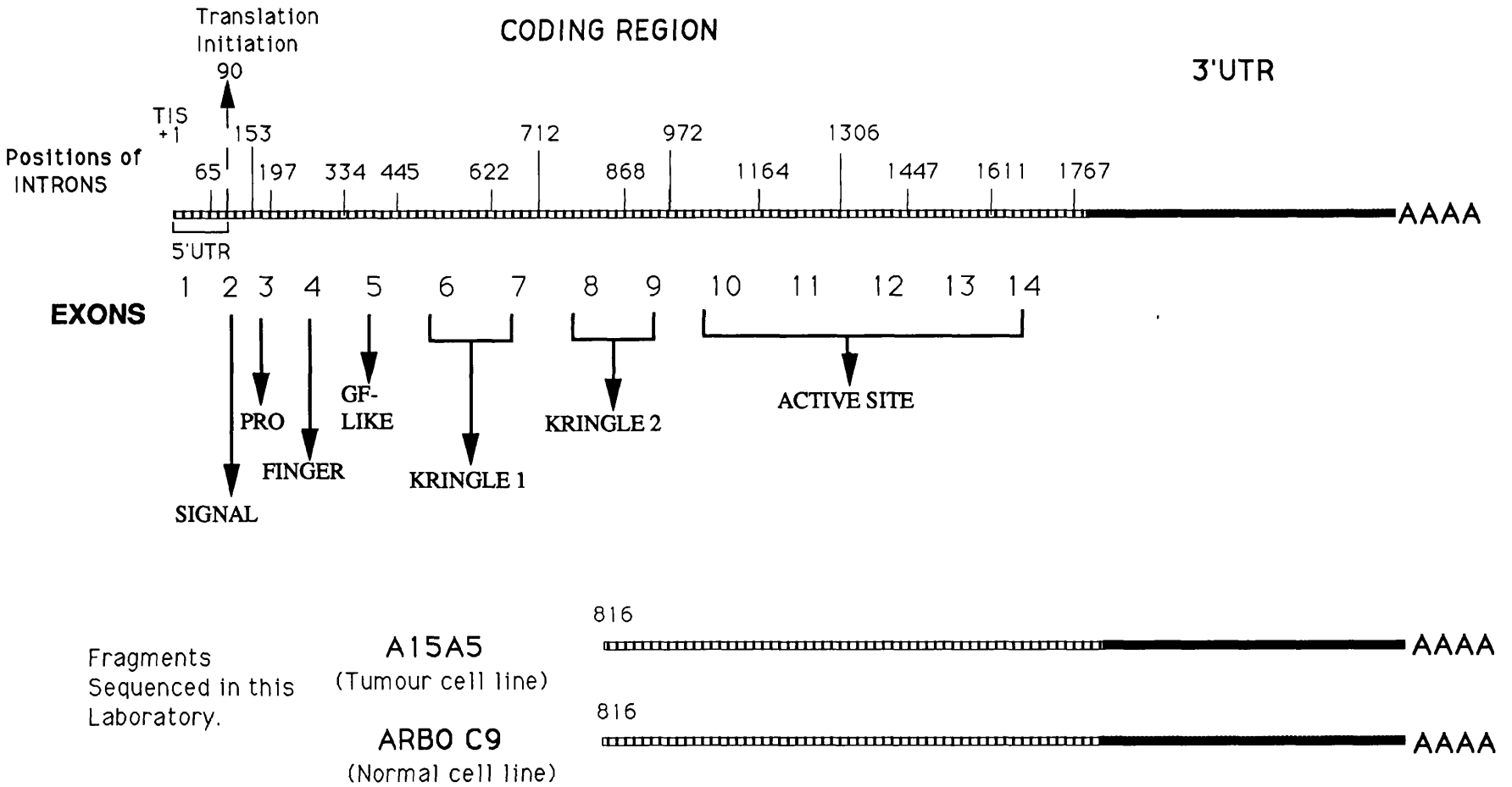


Figure 3.3 : The relationship of tPA fragments sequenced to the published sequence.

However both sequences have regions rich in A and U nucleotides in their 3' untranslated end. These sequences have been demonstrated to have a significant role in the stability of several short lived transcripts. There are 2 AUUUA pentamers in the Acc/Eco fragments of the rat tPA at 2403 and 2419 bases respectively, with a hiatus of 11 nucleotides (figure 3.4)

The ratio of A/U to G/C in the 3'UTR of the tPA gene is 57.89% to 42.11%. However, in the 58 nucleotides between 2371-2448 which contain the 2 AUUUA elements and the longer AUUUUA element there is a ratio of 83.05% to 16.95% respectively. Therefore it is conceivable that this region possessing the reiterated AUUUA pentamer may act as an AU-rich element in the rat tPA message.

(A) : tPA
(2371)AUUUUAAUAUUCGAUGAAAUGACACUAGUAUAUUUAUAUUU
GAAUCUAUUUAGUUUUUACUGUGUUA(2457)

(B) : 1. c-fos
(3970)UUAAUUUAUUUAUUAAGAUGGAUUCUCAGAUAUUUAUUUU
UAUUUUAUUUUUUUCUA(4028)
2. GM-CSF
(638)UAUUUAUAUAUUUAUAUUUUUAAAAUAUUUAUUUAUUU
AUUUAUUUAAGUUCAUAUUCCAUAUUUA(703)
3. IL-3
(736)UAUUUAUUUAUGUAUGUAUGUAUUUAUUUAUUUAUUGCC
UGGAGUGUGAACUGUAUUUAUUUUAGC(810)

Figure 3.4 : (A). The two AUUUA pentamers within the 3'UTR of the rat tPA mRNA.
(B). Three genes which contain multimers of AUUUA in their 3'UTR and have been shown to be regulated through the turnover of their mRNA.

The only difference from the published sequence was found in the coding region of the gene. There is an A to G base substitution at position 1270 in our fragments (position 2081 in Feng). Though this is different to published rat tPA cDNA sequence of Ny et al. (1988), it is same as in the mouse (Rickles et al., 1988), the human (Pennica et al., 1983) the rat tPA genomic sequence of Feng et al. (1990).

3.6 Discussion.

The similarity in size between the rat and the human tPA mRNA, suggests a close relationship between the coding regions of these two genes. The difference in the genomic size of tPA between the species reflect the larger size of the non-transcribed introns and the 3'UTR in man. However coding regions of genes that code for both structurally and functionally significant proteins have close homology or are preserved between species.

This is reflected in the protein and the coding region of tPA mRNA of the human, the rat and the mouse. The rat protein has 92% and 81% homology to mouse and human tPA proteins respectively. The coding region of the rat message has 88.5% and 79.8% homology to mouse and human coding regions respectively, whilst the non-coding 3'UTR has 82.69% homology to mouse 3' UTR. The structural domain of the rat tPA protein is identical to other two characterised mammalian tPA proteins.

The similarities in the protein structure suggest all three

proteins may be regulated in a similar manner. Deviations in the DNA or the mRNA sequences generally point to differences in the regulation of genes between different cells or organisms.

Therefore approximately 1700 bp fragment of the rat tPA cDNA was sequenced from the two cell lines. It was found that the sequence from the two fragments were not only identical but also matched one of the published sequence (Feng et al., 1990) completely. There was a single discrepancy at the position 1270 with the cDNA rat tPA sequence published (Ny et al., 1988). In the Ny sequence there is an adenosine nucleotide at this position whilst in all other published sequences of tPA, including the mouse and the human, a guanosine is found. The G to A transition would cause a glutamic acid to lysine substitution of the amino acid sequence. The difference in the sequence could be a tissue specific difference, as Ny sequence is a combined sequence derived from two cDNA libraries, one from the ovaries and the other a randomly primed library from the rat brain, Feng was a genomic sequence from the rat liver whilst the sequence in this study was obtained from a cDNA library from the rat brain.

However it is also possible that this single difference in the Ny sequence could be an error of sequencing.

Although there was no difference in the sequences, the presence of two AUUUA elements within a region rich in AU nucleotides in the 3'UTR suggest post-transcriptional events

may have an important role in the regulation of tPA mRNA in these cells. The reiterated AUUUA pentamer or closely related sequences have been shown to have a significant influence on both polyadenylation and message stability. There are also several groups of sequences related to the AUUUA outside the AU-rich region of tPA 3' UTR.

These include :

GUUUUUA @ 2171-2177

AUUUUG @ 2080-2085

AUUUUA @ 2034-2039

GUUUUA @ 1951-1956

Several proteins have been identified that have the ability to bind to multimers of AUUUA or to a single pentamer in a U-rich context. Malter (1989) showed oligonucleotides with 4 AUUUA's can form complexes with proteins whilst those without or those with the reverse sequence UAAAU could not.

Several of the proteins that bind to the ARE have been shown to be able to discriminate between different AU-rich sequences. Bohjanen et al. (1991a) identified a constitutively expressed AUUUA binding protein termed the AU-A that bound not only to the canonical AUUUA, but also to AUUUUA and CUUUC. They also identified another protein AU-B that exclusively recognised multimers of AUUUA and had very low affinity to any deviation from this sequence including AUUUUA and AUUA (further discussed in chapter 5).

Therefore those deviations from the canonical AUUUA found in the rat tPA 3'UTR may not be significant, or they may allow alternative methods of regulating the tPA mRNA in different cells.

Although the two mRNA sequences from the tumour cell line and its counterpart showed no discrepancies that could account for the difference in the tPA mRNA levels, the two sequences contain specific sequences in their 3'UTR that could regulate the message level by trans-acting factors. It is also possible that the difference in tPA level is due to differences in the level of transcription of the two genes which is investigated in the next chapter.

CHAPTER 4: TRANSCRIPTIONAL REGULATION OF A15A5 AND ARBO C9

4.1. INTRODUCTION.

Despite the gathering evidence that message stability has a major role to play in regulating mRNA levels, regulation at the level of transcription is the normal method of control of many cellular genes. The level of mRNA can be altered at two stages in the transcriptional process, at the basal level and at the regulated level. The basal level is governed by promoter sequences proximal to the transcription initiation site (TIS), whilst distal sequences called enhancers control the regulated transcription levels (see Kadonaga, 1990 for a review).

The promoter elements are responsible for the assembly of the transcription complex. The consensus sequence TATAAA or the TATA box is the most common transcription initiation promoter sequence in eukaryotes. The transcription complex is assembled at the TATA site which is usually located 25 - 32 nucleotides from the transcription initiation site. The assembly of the complex takes place in a stepwise manner starting with the TATA box factor TFIID and involve incorporating several other transcription factors and the RNA polymerase II.

4.2 Transcription from TATA-less promoters.

Not all promoters contain TATA boxes. Those genes lacking TATA boxes or TATA-less genes can be grouped into two categories depending on the GC content of their promoters. The GC rich promoters generally have multiple transcription initiation sites and several SP1 sites or GC boxes (Ishii et al., 1985), while non-GC rich promoters have one or few transcription initiation sites. The rat tPA gene is classed as a TATA-less gene due to the absence of a canonical TATA box in its promoter (Feng et al., 1990). It can be further categorised as a non-GC rich TATA-less gene due to the low GC content (53%) of its promoter and transcription initiation from a single site. The mouse tPA gene is also a TATA-less gene, but the human tPA gene contain a consensus TATA element (TATAAA). The rat promoter has 78% and 55% homology to mouse and human promoters respectively.

In the absence of a TATA box transcription initiation of the rat tPA gene has been found to take place through the SP1 elements in its promoter (figure 4.1). Results from *in vitro* studies suggest that SP1 elements may act as transcription initiation elements in the rat tPA promoter. Ohlsson et al. (1993) demonstrated that the two SP1 sites proximal to the TIS are required for the constitutive expression of tPA in the rat neuroblastoma cell line B103 and for FSH mediated induction of the gene in rat granulosa cells. They also found inactivation of the GC boxes almost abolishes the tPA promoter activity in these cell lines. Furthermore, in the murine tPA promoter

which is also a TATA-less gene, the SP1 sites were also shown to be essential both liver and brain specific expression of tPA (Pecorino et al., 1991). Therefore the evidence points to the SP1 sites adjacent to the TIS acting as the transcription initiation sequence in the rat gene.

Another element proposed to initiate transcription from non-GC rich TATA-less promoters is the initiator element (Inr), originally descried by Smale & Baltimore (1989). The 17 bp Inr of the mouse TdT gene which traverses its transcription initiation site was found to regulate the basal level of transcription of the gene. Recombinant clone studies showed that the Inr could be strongly activated by a TATA box or by a heterologous promoter in the absence of a TATA box. Furthermore, upstream promoter elements were also found to directly activate Inr mediated transcription while mutations within the Inr element affected the efficiency of its initiation. Therefore these authors proposed that the Inr as the minimal sequence element that can initiate transcription from a gene.

The promoter sites of the adenovirus major-late promoter (AdML), rabbit ß-globin and human immunodefficiency virus 1 which initiate transcription from inactive TATA-like sequences have been found to have homology to the mouse TdT Inr. However, there is no homology between the Inr and the rat or mouse tPA start sites, but it is possible there may be different types of Inr elements in different promoters which can initiate

transcription in association with upstream sequence elements.

4.3 The role of enhancers in transcription regulation.

The rest of the promoters of rat and mouse tPA genes also differ in their potential binding sites for transcription factors. The rat gene has one AP1, one CTF/NF1, one CRE and three SP1 sites (inclusive of the initiation element) in its promoter (figure 4.1), while the mouse tPA promoter consists of five AP2s, one SP1, one TRE and one CTF/NF1 site.

Transcription factors (TFs) that bind to these elements interact with the transcription complex assembled at the TIS to alter the level of transcription of the message. Of the several models put forward to explain the interactions between the factors bound to the distal enhancer elements and those bound to the proximal promoter elements the looping model is the most widely accepted theory now. This model suggest that once the TFs have bound to the enhancer elements the DNA folds (loops-out) to bring these into close contact with the basal regulatory factors. Several variations to this model has also been proposed (Ptashne & Gann, 1990) (figure 4.2).

The regulatory factors can be grouped into universal factors e.g. SP1, which is expressed ubiquitously by many cells and tissue specific factors, e.g. Oct 1 which is expressed in a tissue specific manner. Although SP1 can be classified as a universal factor as binding sites for the factor is present in many promoters, it may not be always involved in initiation of gene transcription

as found with tPA.

The affinity of some promoter elements to multiple transcription factors leads to their genes being regulated in disparate manner in different tissue. For example, the PEA3 enhancer element in the uPA promoter has been shown to influence uPA gene transcription in a different manner depending on the cell line. Nerlov et al. (1991) studied PEA3 mediated uPA expression in three human transformed cell lines; PEA3 was found to induce highest levels of uPA in HepG2 hepatoma cells, it was less active in the HT 1080 fibrosarcoma cell line and was inactive in the third, HeLa cell line.

The murine tPA gene can also be expressed in a tissue specific manner (Pecorino et al., 1991). These workers used an *in vitro* assay to investigate the effects of murine brain, kidney and liver extracts on the expression of tPA mRNA. The brain had the highest steady state levels of tPA mRNA while the kidney had lower levels and it was undetectable in the liver. *In vitro* transcription assays using extracts from the three sites showed highest levels of transcription from the brain. There was lower levels of transcription in the presence of kidney extracts and even lower but detectable transcription in the liver. These data suggest that transcription rates could partly account for the differences in the tPA levels between the three sites. Furthermore, mobility shift assays during this study found specific brain derived factors that had increased affinity to the GC boxes or to sequences overlapping the GC

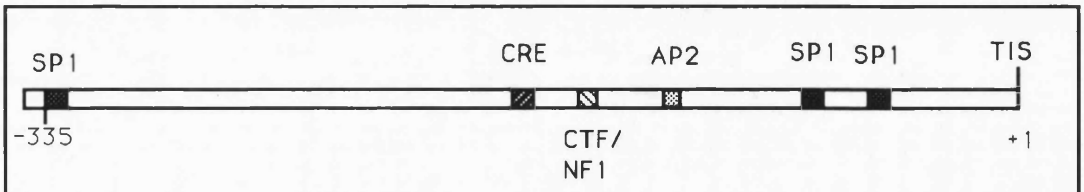


Figure 4.1 : Regulatory elements in the rat tPA promoter

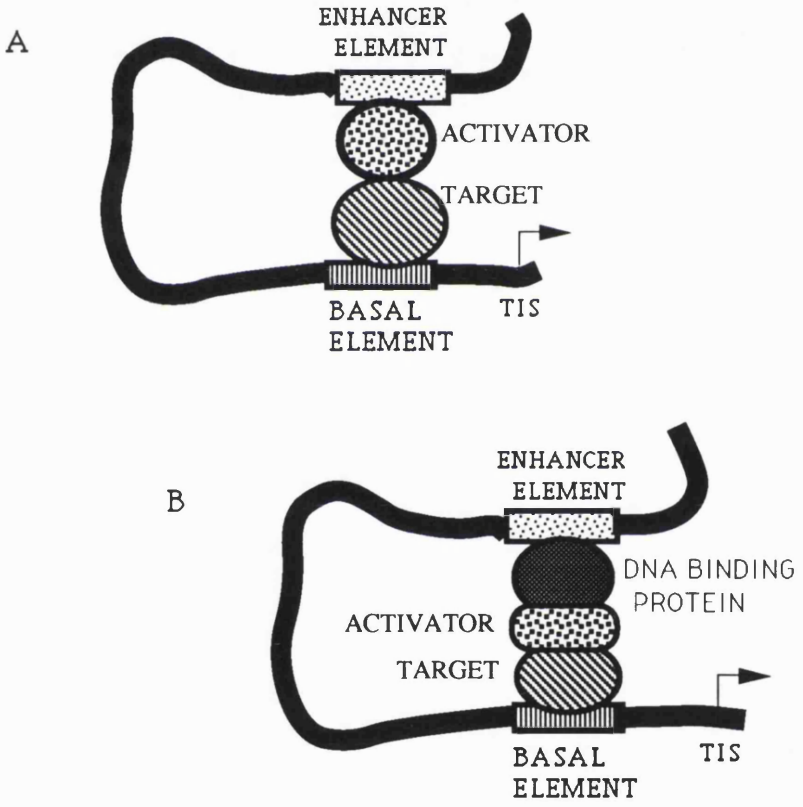


Figure 4.2 : Proposed variations of the looping model*.
A. Direct interaction between the activator and the universal target protein. The activator possesing both DNA and target protein binding sites.
B. The target binding and enhancer binding activity in different factors.
* adapted from Ptashne and Gann (1990).

boxes of the tPA promoter. Therefore it was suggested that the brain contain specific factors that can regulate brain specific tPA transcription.

Based on this model it could be suggested that the difference in the tPA message level between the A15A5 glioma and the normal ARBO C9 brain cell line may be a transcriptional difference, possibly due to differential expression of brain specific transcription factors between the cell lines.

4.4 The nuclear run-on transcription assay and the measurement of RNA transcription.

Nuclear run-on transcriptions assays were used in this study to determine whether the constitutively high mRNA level in the A15A5 cell line is due to transcriptional activation of the tPA gene. Results in the previous chapter showed no differences in the tPA fragments sequenced that could alter stability of the tPA message in the two cell lines. Therefore it is possible that expression of tPA in these cell lines are regulated through differential transcription. As both these cell lines are brain derived there may be differential expression of brain specific factors as suggested by Pecorino et al. (1991).

The nuclear run-on assays are transcription assays carried out *in vitro* using native nuclei from the cells in which the gene being analysed belong to. In nuclear run-ons no new transcripts are initiated, but those already initiated transcripts present at the time of isolation of the nuclei are faithfully extended using radiolabelled nucleotides. The labelled transcripts are then isolated and hybridised to slot blots carrying the sequences to be analysed.

As the rate of transcription is governed by the rate of initiation of transcription, run-ons provide a suitable technique to determine differences in transcription rate between genes.

There are several advantages for using run-on assays to

measure transcription instead of other *in vitro* transcription assays. As nuclear run-ons are carried out in isolated nuclei, the cell's nucleosomal structure remains intact and transcription is carried out by native enzymes and transcriptional machinery. During other *in vitro* assays, either recombinant templates or exogenous extracts are used to transcribe genes. Therefore they do not always reflect the *in vivo* state of transcription of the gene. As run-ons are carried out in nuclei, the rates of transcription measured resemble natural state of transcription in the cell.

There are several disadvantages of using run-ons to measure transcription. Nuclei by nature are fragile and have a short viability outside their natural environment. Therefore active transcription diminishes within 20-30 minutes in isolated nuclei, thus not only do gentle isolation methods have to be used, but the assay also has to be optimized for speed of isolation.

4.5 RESULTS

Although methods for several run-on assays have been published for different cell systems no general protocol exists indicating the need to adapt each assay to reflect the distinctiveness of each cell system. Initial run-ons in this study were carried using an assay based on Greenberg and Ziff (1983). Although there was active transcription in the cells, as seen by the transcription of high copy number rat repetitive sequences, transcription of tPA mRNA could not be detected in these assays.

Therefore the protocol had to be extensively modified to improve its limits of detection in these cell lines. The modifications were made to all of the major steps of the assay, i.e. the method of isolation of nuclei, the transcription reaction and the method of isolation of the labelled transcripts. The mode of isolation of the nuclei was altered to increase the speed of isolation. The concentration of the detergent (NP40) and the volume of cell lysis buffer used in the original assay did not lyse the cells sufficiently or rapidly enough. Therefore the detergent concentration was raised to 0.75% and the cells were lysed in an increased volume (4mls) of buffer .

In the original assay transcription elongation was allowed to proceed at 37°C for 1 hour. However, others including Marzluff and Huang (1984) have established that a lower temperature between 27°-30°C provides the optimal temperature for

transcription in nuclear run-ons, with no measurable increase in incorporation of label taking place beyond 25 minutes. Therefore in these run-ons transcription was carried out at 28°C and the reactions were terminated after 25 minutes.

The third major change from the original procedure was in the method of isolation of the labelled transcripts. Instead of the common but cumbersome procedure of filter disc immobilization and TCA precipitation of newly labelled transcripts, the method of Celano et al. (1989) which itself is based on the original Chomczynski and Sacchi (1987) method was used to isolate labelled RNA. Together these modifications considerably reduced not only the time taken to carry out the procedure, but also improved the sensitivity of the assay.

The signals were standardised by hybridising equal number of counts of labelled RNA to DNA blotted on to nitrocellulose using a slot blot apparatus.

Figure 4.3 shows the level of transcription of tPA between the tumour A15A5 and the normal ARBO C9 cell lines 4 hours after addition of fresh medium to exponentially growing cells. Densitometric analysis show the tPA gene to be transcribed at an approximately 1.5 -fold higher level in the glioma cell line than in the normal brain cell line (data not shown).

The level of transcription at 24 hours after medium change (figure 4.4) was not significantly different, being about 1.7 fold

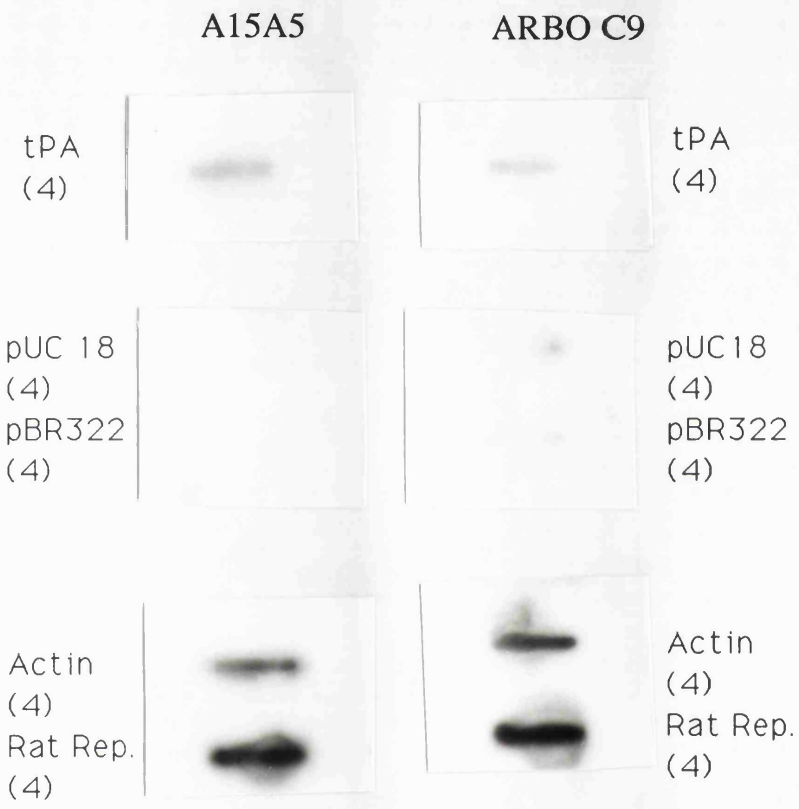


Figure 4.3 : The rate of transcription of tPA in A15A5 and ARBO C9 at 4 hours after change of medium.

( ) = µgDNA/ slot

higher in A15A5 than in ARBO C9. Results from several run-on assays showed that the difference in constitutive level of transcription between A15A5 and ARBO C9 to be always less than 2 -fold.

Several control sequences were used in the slot blots to obtain an indication of the level of transcription and to confirm that there is no spurious binding to unrelated sequences. There were strong signals for the high copy genes actin and rat repetitive sequences as expected. The rat repetitive sequences used in these slot blots were brain specific 4D12 sequences (Witney and Furano, 1984). There was no signals for the bacterial sequences pUC18 and pBR322.

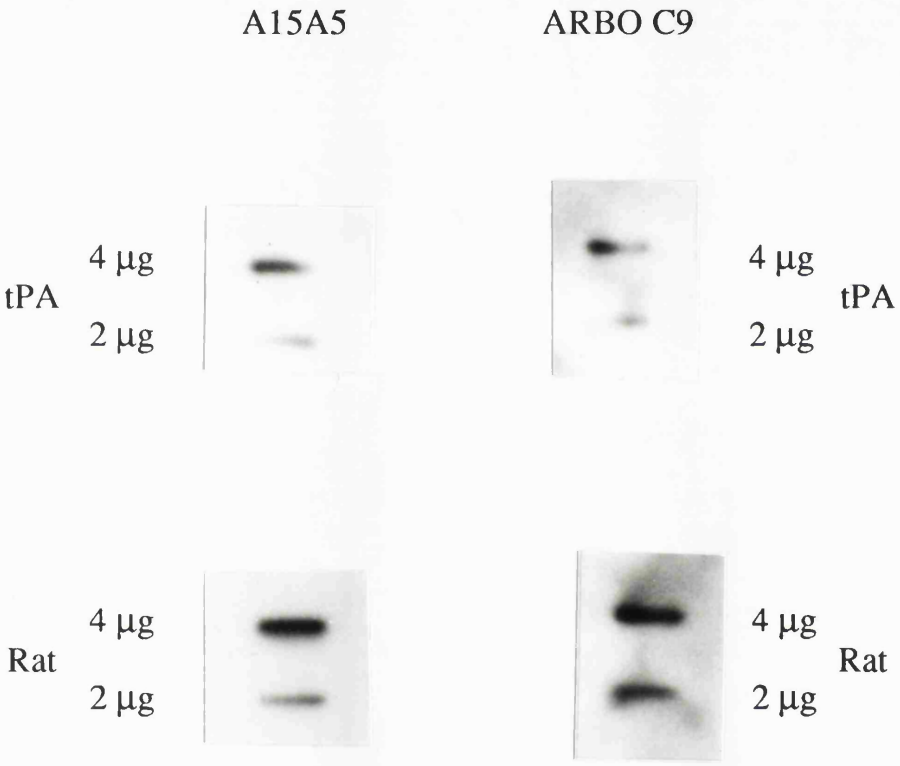


Figure 4.4 :The rate of transcription of tPA in A15A5 and ARBO C9 at 24 hours.

Rat = rat repetitive.

4.6 DISCUSSION.

Nuclear run-on transcription assays were carried out to determine the rate of transcription of the tPA gene in the A15A5 glioma cells and the normal ARBO C9 cells. As nuclear run-ons are carried out in isolated nuclei they provide a truer picture of transcription rates than other *in vitro* transcription assays. Several important modifications were made to the original assay used for these run-ons to improve its sensitivity. Key modifications include increasing the concentration of the detergent (NP40) used to lyse the cells to 0.75%, the volume of cell lysis buffer and the concentration of ribonucleotides, lowering the length of time of the transcription reaction and altering the method of isolation of labelled RNA.

The multitude of run-ons carried out in this study indicated several important caveats about these transcription assays. Principally there is a limit to the sensitivity of nuclear run-ons, specially for detecting low copy number genes. Furthermore, RNAse free conditions and the speed of isolation of the nuclei are crucial. The longer the time spent on isolating the nuclei, the less active were the nuclei during transcription assays. There was not much difference in the level of incorporation of radiolabel into RNA at 4 or 24 hours after change to fresh medium, which indicate that total level of transcription remain high in these cells throughout the cell cycle.

Previous studies established that the steady state level of tPA

mRNA in the glioma cells is 10-20 fold higher than the normal counterpart. Several workers have demonstrated that the tPA gene can be transcriptionally activated under certain circumstances. Pecorino et al. (1991) found that the murine brain posses constitutively higher level of tPA than the liver. This elevated level was found to be primarily due to the increased transcription of the tPA gene in the brain. Meanwhile Ohlsson et al. (1993) have described using nuclear run-on assays that the constitutively elevated tPA mRNA level in the rat neuroblastoma cell line B103 is due increased transcription of the gene compared to that in the primary cultures of rat granulosa cells. However it has to be pointed out that the two cell lines in the above study are of different origins.

The results from this study shows that the tissue-plasminogen activator gene is transcribed at a similar rate between the glioma cell line and the normal cell line and cannot account for the constitutively higher level of tPA message found in the neoplastic cells.

The difference in the level of transcription at 4 and 24 hours were also identical between the cell lines. This suggest that tPA is transcribed continuously in both cell lines. Despite the similarity in the level of transcription it is difficult to detect tPA message in total RNA from ARBO C9 cells, which suggest that there is considerable differences in the regulation of degradation or the stability of the tPA message between the

two cell lines. The cDNA fragments of tPA sequenced from the two cell lines include the 3'UTR which is proposed to contain many of the sequences that regulate message stability. Although no differences were found between the two sequences, this does not mean that message stability could not be involved in regulation of tPA mRNA levels in these cell lines as degradation involves not only specific sequence elements but also trans-acting factors that bind to these elements. Therefore in the next chapter stability of the tPA message in the A15A5 cell line was determined.

CHAPTER 5 : STABILITY AND CELLULAR LOCALIZATION OF tPA mRNA.

5.1 INTRODUCTION

In chapter 4 sequence elements involved in regulating gene transcription was discussed. After the message is transcribed it undergoes several post-transcriptional processes. These include removal of introns, addition of the cap structure to the 5' end and the poly-A tail to the 3' end, transport to the cytoplasm, translation and finally degradation of the message. Regulation of the mRNA stability and thus the message available for translation provide a considerable degree of flexibility in manipulating gene expression, as small changes in transcription can be amplified through altered degradation of the message.

Several elements, both cis-elements and trans-acting factors are involved in regulating mRNA stability (partly discussed in chapter 3).

5.2 The role of the poly-A tail in regulating mRNA stability in somatic cells.

One of the important elements is the poly-A tail, which has been shown to regulate translation activation of deadenylated latent tPA mRNA during oocyte maturation. In somatic cells however, deadenylation of the poly-A tail usually leads to degradation of the message. Evidence for this process has been obtained by several groups of workers including Nudel et al. (1976) who showed that rabbit ß-globin mRNA with a tail of 32 poly-A

nucleotides has the same functional stability as a message with 150 nucleotides. When the tail is deadenylated to 16 residues the stability of the mRNA decreases by 10-fold or close to the fully deadenylated state. Generally messages with less than 30 nucleotides in their poly-A tail are rare. Whether at this tail length the deadenylation stops or the transcripts undergo immediate degradation is yet to be elucidated, but this data suggests that deadenylated mRNAs are generally unstable. That deadenylation is the preliminary event of message degradation has been demonstrated for several transcripts including c-fos and c-myc (Wilson and Treisman, 1988).

The close link between poly-A removal and message degradation, the existence of specific signals for cleavage, poly-adenylation, and deadenylation suggest that this is a regulated event modulated through trans-acting factors. A protein that bind to the poly-A tail was first demonstrated by Sachs et al. (1986). The protein termed the poly-A binding protein (PABP) has been characterised and found to be remarkably conserved from yeasts to humans (Sachs and Davis, 1989). It is a protein of 577 and 633 amino acids in yeast and human respectively. The protein consists of two domains. The N-terminal domain which has homology to other RNA binding proteins is thought to be important for RNA binding function. This domain has also been proposed to be responsible for the ability of PABP to migrate from one poly-A to another. The C-terminal domain is thought to interact with other cytoplasmic factors that regulate message stability.

Experiments that have established PABP as having an essential function in mRNA stability include work by Brewer and Ross (1989), who found PABP could regulate degradation of ß-globin mRNAs possessing poly-A tails but not those lacking a tail.

While competition experiments with ß-globin mRNA demonstrated that there is a 7-fold rise in its degradation in the presence of competitor poly-A polymers, but not in the presence of poly-C, poly-G or poly-U sequences (Bernstein et al., 1989a, 1989b) These data imply that binding of PABP protects poly-A tails from deadenylation, possibly through shielding from being attacked by poly-A nucleases.

Although PABP has a role in mRNA stability it is not the only trans-acting factor that is involved in regulating message degradation as different messages can be degraded at different rates. Therefore mRNA degradation may involve PABP as well as other mechanisms which act through other signals in the mRNA molecule.

5.3 The function of A and U nucleotide rich elements in RNA degradation

The AU-rich sequences are known to mediate message stability (briefly discussed in chapter 3). The AUUUA pentamer is present in the 3' UTR of many unstable transcripts such as c-fos, c-myc and GM-CSF (granulocyte macrophage-colony stimulating factor) and has been shown to impose instability to those messages carrying it.

Wilson and Triesman (1988) showed deletion of the 3' ARE from c-fos leads to the mutant mRNA becoming more stable than the short lived wild-type c-fos mRNA. Whilst transfer of the GM-CSF ARE containing several AUUUA elements to the 3' UTR of the more stable ß-globin message leads to a decrease in the ß-globin half-life from 17 hours to 30 mins. An identical construct but with disrupted AUUUAs does not depress the ß-globin half-life as much. *In vitro* transcription assays showed that transcription does not account for this effect as both constructs had similar transcription rates (Shaw and Kamen, 1986). Furthermore Brewer and Ross (1988) demonstrated that endonuclease cleavage of c-myc mRNA takes place immediately 3' of its AUUUA element.

5.4 Trans-acting factors that bind to AU-rich sequences.

Several AU binding factors have now been identified by different workers varying in size from 30 to 70 kDa . Malter (1989) identified a protein complex (AUBF) that binds to oligonucleotides with 4 AUUUAs, but not to identical oligonucleotides lacking the pentamers or containing the inverted sequence UAAAAU. Vakalapoulou et al. (1991) have identified a 32 kDa nuclear protein in HeLa cells that can bind to the ARE of GM-CSF 3' UTR, while Brewer (1991) using an in vitro assay system have identified a trans-acting factor (AU-F) that bind to the c-myc ARE and accelerate its turnover.

Furthermore, Bohjanen et.al. (1991a,b) found that T cells synthesize two closely related cytoplasmic factors termed AU-B and AU-C that specifically bind to AUUUA pentamers of GM-CSF,

IL-2 and TNFα but not to the AU element of c-myc.

Several of the factors, though identified in different cell systems have similar molecular weights and binds to identical sequences suggesting that they may be similar factors or belong to the same family. Evidence suggest that AU-A (Bohjanen et al. 1991a), AUBP (Vakalapoulou et al. 1991) and AUBF (Malter 1989) may be identical factors. All are found abundantly, perhaps constitutively expressed and all recognise similar 3' UTRs (table 5.1). There are however differences within this group, AUBP and AU-A are predominantly nuclear while AUBF is cytoplasmic.

Some of these factors (eg AUBP) can bind not only to reiterated AUUUA elements, but also to a single pentamer when it is in a U-rich context (Vakalapoulou 1991, Brewer 1991) which suggest that the wider AU-rich region has an important role in the binding of these proteins to 3' UTRs.

Recent evidence indicates that some of these factors are related to the heterogeneous nuclear ribonuclearproteins (hnRNP). These are a family of abundant nuclear proteins that are involved in RNA metabolism at the level of pre-RNA processing. All of these proteins contain RNA binding domains (Dreyfuss et al,1988). A 38 kDa protein associated with cytoplasmic poly-A mRNA in HeLa cells has been identified as the hnRNP A1 protein. This protein was shown to be able to shuttle between the nucleus and the cytoplasm with increased accumulation in the cytoplasm after transcription inhibition (Dreyfuss et al., 1984).

Table 5.1: Several of the AU-rich element binding proteins*

AU binding protein	Source	Size kDa	Cellular location	Binding mRNA	Reference
1. AUBF	Human peripheral blood mononuclear cells	36	cytoplasmic	IL, GM-CSF, TNF,v-myc, c-fos.	Malter, 1989.
2. AU-A	Human T-lymphocytes	34	predominantly nuclear	IL2, GM-CSF, c-myc, TNFα	Bohjannen, 1991,1992.
3. AU-B	" "	30	cytoplasmic	IL2, GM-CSF, TNFα, but not c-myc	" "
4. AU-C	" "	43			" "
5. AUBP	HeLa cells	32	nuclear and cytoplasmic	GM-CSF, c-myc,c-fos.	Vakalapoulou, 1991.
6. AUf	Erythroleukaemic cell line	37 & 42	cytoplasmic	GM-CSF, c-myc.	Brewer, 1991.

* This is not a comprehensive list of AU-binding proteins, but an indication of some of the proteins studied so far.

Hamilton et al. (1993) identified five different cytoplasmic factors in human peripheral blood lymphocytes that bind to transcripts with ARE elements. Several of these were found to correspond in size to other previously identified factors. Antibody studies using antibodies to hnRNP A1, C1 and C2 showed that at least three of these factors are homologues of hnRNP factors. That is, antibodies to hnRNP A1 bound to the 36kDa factor, while antibodies to hnRNP C1 and C2 bound to 43 and 36 kDa factors respectively. Transcription inhibition by actinomycin D was found to elevate the levels of 36 kDa protein, similar to the results found by Dreyfuss et al. for their 38 kDa factor which was also a homologue of hnRNP A1. Translation inhibition by cycloheximide was found to have no effect on the 36 kDa protein.

5.5 The effect of transcription and translation inhibition on mRNA stability.

However inhibition of translation is known to stabilise various mRNAs including uPA (Altus and Nagamine 1991), histone (Stimac et al., 1984), tubulin (Baker et al., 1986), GM-CSF (Thorens et al., 1987) and EGF receptor mRNA (Kesavan et al. 1990). Several hypotheses have been put forward to explain the mechanism of action of translation inhibition on mRNA stability. These include, 1. degradation of message due to the presence of a labile RNase which disappears rapidly following inhibition of translation, eg c-fos mRNA half life increases to several hours upon protein synthesis inhibition (Wilson and Triesman, 1988).

2. Superinduction due to translation inhibition, seen with immediate early (IE) gene transcripts suggesting that transcription shut-off of these genes require active translation. 3. The labile repressor hypothesis suggest some genes eg c-fos, is kept inactive due to continuous synthesis of a labile repressor if transcription which disappears upon translation inhibition. But later it was shown using run-ons that transcription of c-fos takes place in the presence of cycloheximide but not in the presence of another translation inhibitor puromycin, thus discounting this theory of a labile repressor (Edwards and Mahadevan, 1992), as translation arrest itself is not sufficient to initiate transcription of c-fos and c-jun.

This body of work suggest that both transcription and translation inhibition can influence mRNA levels possibly through modulating trans-acting factors that bind to ARE sequences in transcripts. Therefore the effect of inhibition of both transcription and translation on the stability of the tPA mRNA in the two cell lines were studied. Earlier work indicated no differences in the sequence of a 1700 nucleotide fragment of the tPA cDNA between A15A5 and ARBO C9 (Chapter 3) and that tPA mRNA is transcribed at a similar rate in the cell lines (Chapter 4).

The 3' UTR sequence of the tPA cDNA from both cell lines was shown to possess two AUUUA pentamers within a region rich in AU nucleotides (chapter 3). The reiterated AUUUA has been demonstrated to be involved in the regulation of stabilities of mRNAs possessing this sequence in their 3'UTR. Several proteins

have been isolated from both the nucleus and cytoplasm of cells that have the ability to bind to this sequence and alter the stability of mRNA. Therefore it has been suggested that some of these factors may be involved in regulating nuclear levels of certain RNAs (Vakalapoulou et al., 1991).

Thus to determine whether the increased stability of tPA mRNA is a nuclear or a cytoplasmic event, cellular distribution of the tPA mRNA in the two cell line was also investigated.

During this study the expression of mRNA was measured under two conditions; in the presence of serum and in the absence of serum.

The mRNA levels in the presence of 15% serum represent the expression of the message during normal growth conditions. However, collection of conditioned medium (CM) to measure plasminogen activator (PA) activity is usually carried out in the absence of serum, as it was demonstrated as long ago as 1973 that serum has an inhibitory effect on PA mediated fibrinolytic activity (Unkeless et al., 1973, Jones et al., 1975). It has been proposed that serum may contain or induce specific and non-specific inhibitors of PA activity. Furthermore it is also possible that serum may influence regulation of mRNA levels as it has been demonstrated by Berger et al. (1991) that serum could elevate levels of PABP. This is a trans-acting factor that bind to poly-A tails of mRNA and has been proposed to be involved in regulating poly-A removal and degradation of certain transcripts.

Therefore the tPA mRNA in the presence of serum was determined to analyse the expression during normal growth conditions. While the levels in the absence of serum relates to the expression of mRNA during the collection of serum to measure the PA activity of the cells. The CM is usually collected over 24 hours.

5.6 RESULTS

5.6.1. The affect of transcription inhibition on tPA mRNA stability.

Actinomycin D was used as an inhibitor of mRNA transcription to analyse its effect on the stability of tPA in A15A5 glioma cells. The stability of the message in ARBO C9 cells was not determined as tPA mRNA in these cells are expressed at too low a level to be investigated.

Actinomycin D acts by binding to the DNA template and inhibiting RNA polymerase II from elongating nascent transcripts. In eukaryotes translation of proteins carry on for several hours after transcription inhibition, therefore the cells remains viable for a certain time period after the addition of the inhibitor. However high concentrations of the drug can be toxic to the cells. Therefore it is important to ascertain the optimum concentration that causes transcription inhibition without effecting the viability of the cells. The incorporation of

^{3}H (tritiated) uridine by cells in the presence of actinomycin D was determined to identify the level of transcription inhibition at different concentrations of the drug.

The usual concentration of actinomycin D used to inhibit transcription is 5ug/ml. A pilot study carried out using 2.5, 5 and 10 ug/ml actinomycin D showed a rapid inhibition of ^{3}H-uridine incorporation into the cells. However upon observation of cells under the light microscope for changes in morphology, it was found that actinomycin D at concentrations above 2.5 ug/ml were toxic to the cells (table 5.2). At 2.5 μg the normal morphology of the cells alter by 2 hours and soon they begin to detach from the substratum indicating that they have become non-viable due to the toxicity of the drug.

At lug/ml although there was some change in morphology there was no gross detachment of cells over the 24 hour period observed. Also, this concentration caused >90% inhibition of transcription by 1.5 hrs and >95% by 4 hrs (figure 5.1). Therefore it was decided to use lug/ml actinomycin D to inhibit transcription in these cells.

Actinomycin D was administered to cells growing in the exponential state in 15% foetal calf serum. At the time of treatment with the inhibitor the growth medium was replaced

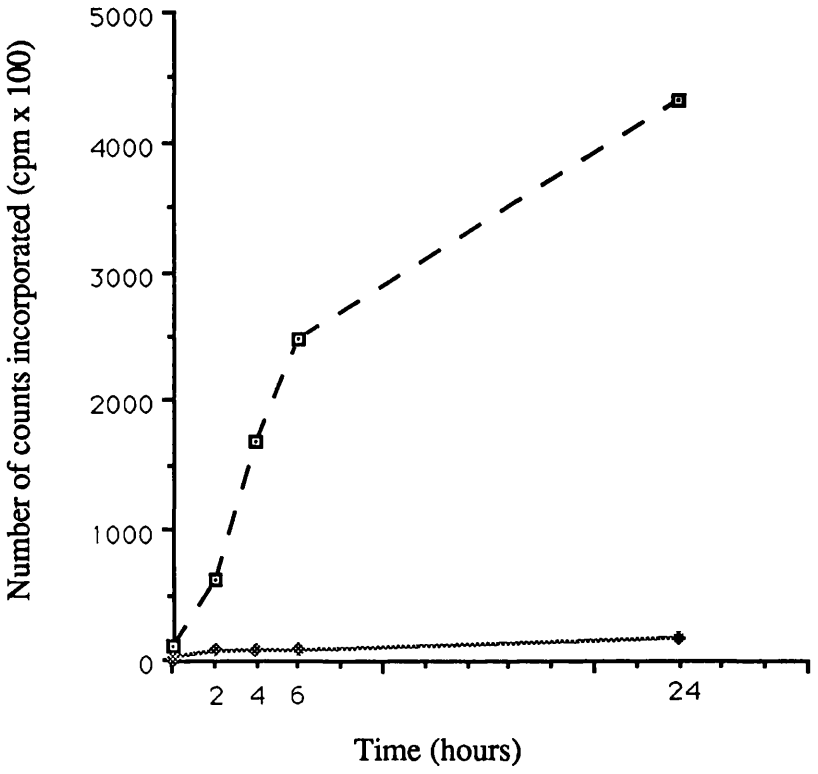


Figure 5.1 : The level of incorporation of ^{3}H uridine in to A15A5 cells.

..... Actinomycin D (1µg/ml)

— — — Control

TIME hours	Actinomycin D (µg/ml) CONTROL	0.1	0.5	1.0	2.5	5.0
2	Settled	Settled	Settled.	Starting to round up	Starting to round up	Starting to round up
3.5	Settled	Majority as control, a small minority rounded.	Few have rounded.	Many rounded	Many rounded, few detached.	Becoming detached.
4.5	As above	More have rounded.	More rounded	As above	As above	Many have detached
6.5	Completely setteled.	More rounded.	As above	Majority rounded, but not as much as at 2.5 µg/ml; some detached	Majority rounded, many have detached	Majority detached.
22	Divided overnight	Majority rounded, few detached.	Majority rounded, few detached	As 0.5µg/ml, some detached.	All rounded, majority detached.	As above.

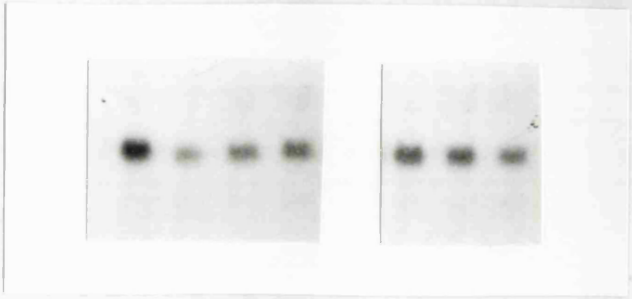
Table 5.2: The effect of actinomycin D on the viability of A15A5 cells.

with fresh medium. Total RNA was isolated at specific time intervals and was analysed on northern blots and probed with the 1700 bp insert already sequenced and confirmed as tPA in chapter 3. To measure RNA levels in the absence of serum, the medium at the time of addition of the drug was replaced with medium lacking in serum or Dulbecco's modified eagles medium (DMEM) alone. The blots were stripped and reprobed with ß-actin to standardise the message levels.

The results of inhibition of transcription in the presence of serum is shown in figures 5.2a and 5.2b. The figures show the level of tPA mRNA at different time intervals, the level of ß actin in the cells and a graph of tPA mRNA equilibrated to the ß-actin signal.

The normal time course indicated that the tPA mRNA level rises over the 6 hour period observed. While the time course after inhibition of transcription by actinomycin D show that the message level remain constant over this period. That is, there is no gross destruction of the message or the tPA mRNA is stable in A15A5 cells under normal growth conditions.

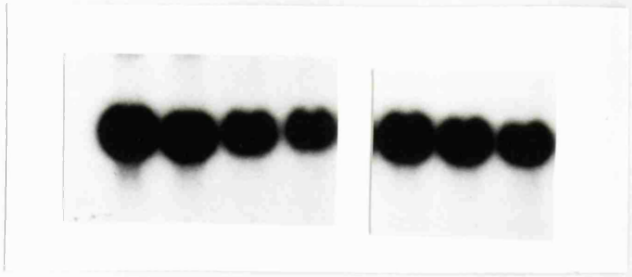
The figures 5.3a and 5.3b show the tPA mRNA level in the absence of serum. In cells untreated with actinomycin D the mRNA level rises over 6 hours. This is not as rapid a rise as in the presence of serum, but it was found in other experiments



0 2 4 6 2+ 4+ 6+

Time (hours)

tPA mRNA



0 2 4 6 2+ 4+ 6+

Time (hours)

Actin mRNA

Figure 5.2a: A time course of tPA mRNA after inhibition of transcription in the presence of serum.

+ = actinomycin D

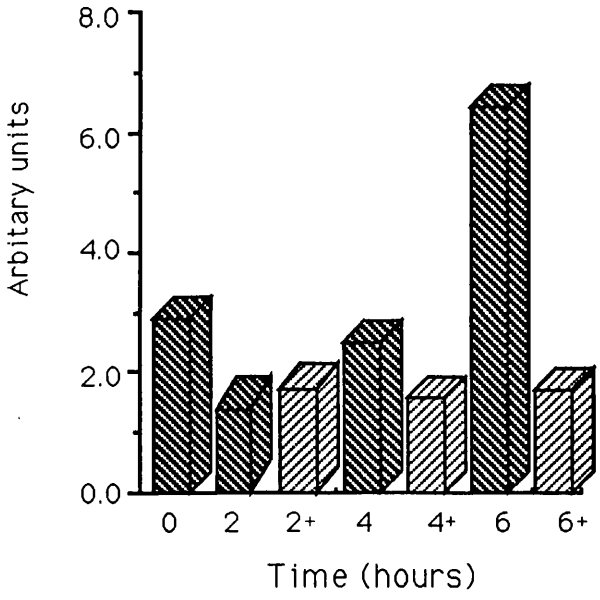
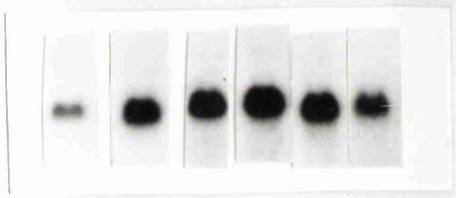
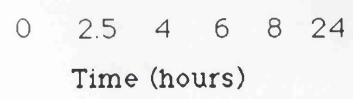


Figure 5.2b :The graph of tPA mRA level from figure 5.2a standardised to ß-actin message level.

Control (15% serum)

Actinomycin D





tPA

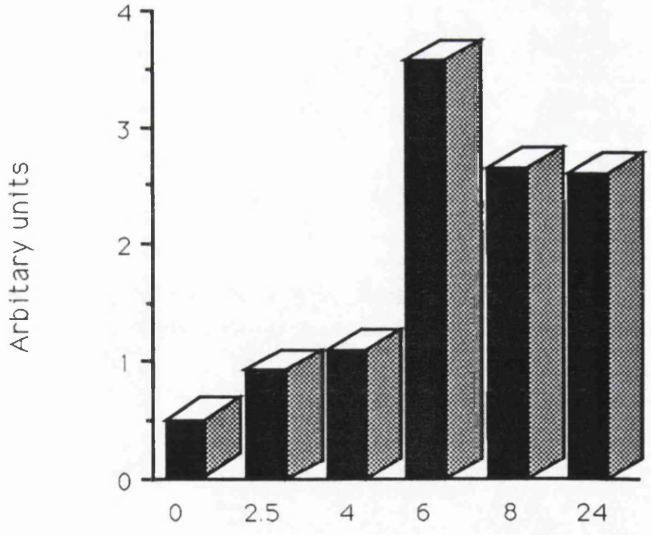
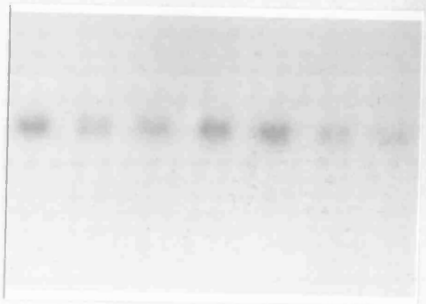


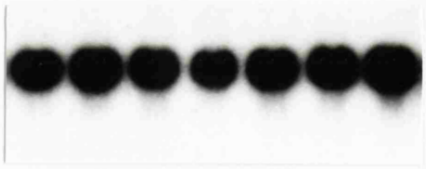
Figure 5.2c : Expression of tPA mRNA in A15A5 cells over 24 hours in the presence of serum.



6 4 2 0 2+ 4+ 6+

Time (hours)

tPA



6 4 2 0 2+ 4+ 6+

Time (hours)

Actin

Figure 5.3a : The time course of tPA mRNA after inhibition of transcription in the absence of serum.

+ = actinomycin D

including that represented in figure 6.2, the increase continues over 24 hours and attain a mRNA level similar to that in the presence of serum at 24 hours. The message level in the presence of serum usually peaks between 6 - 8 hours and decreases slightly by 24 hours (figures 5.2c, 5.5 and 6.4). The other time courses in this study (figure 6.2 and 6.5) also indicated a longer lag before mRNA levels begins to rise in the absence of serum. However at 24 hours the tPA message levels are comparable under both conditions (figure 6.1).

Inhibition of transcription in the absence of serum show a modest decline of the tPA mRNA during the 6 hour period, suggesting that the transcript is less stable compared to that in the presence of serum. However the message level remains detectable and substantial during this period.

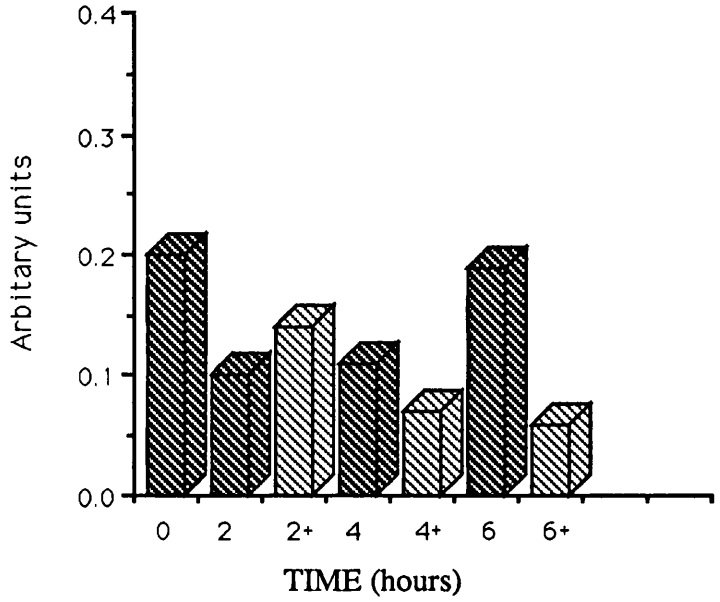


Figure 5.3b :The graph of tPA mRA level after inhibition of transcription in the absence of serum standardized to ß-actin message levels.

Actinomycin D

Control (DMEM)

5.6.2 The effect of translation inhibition on tPA mRNA levels in A15A5

To determine whether inhibition of translation leads to the stabilizing of tPA mRNA in A15A5 cells, message levels were determined in the presence of the translation inhibitor cycloheximide.

Cycloheximide acts by trapping the RNA strand on polysomes, and thus inhibits peptide chain elongation. The usual concentration of cycloheximide used to inhibit translation is 10 ug/ml. To determine whether this concentration is detrimental to their viability, the cells were observed in the presence of 10 ug/ml cycloheximide. There was no large scale detachment from the substratum or an alteration in the morphology of cells over a 24 hour period observed, indicating that these concentrations are not toxic to the cells and that they remain viable over this time period.

The level of incorporation of ^{35}S methionine in the presence of 10 ug/ml of cycloheximide was determined. This concentration was found to be sufficient to inhibit translation by >95% in these cells (figure 5.4)

To determine the affect of translation inhibition on the tPA mRNA cells growing in the log phase were treated with 10

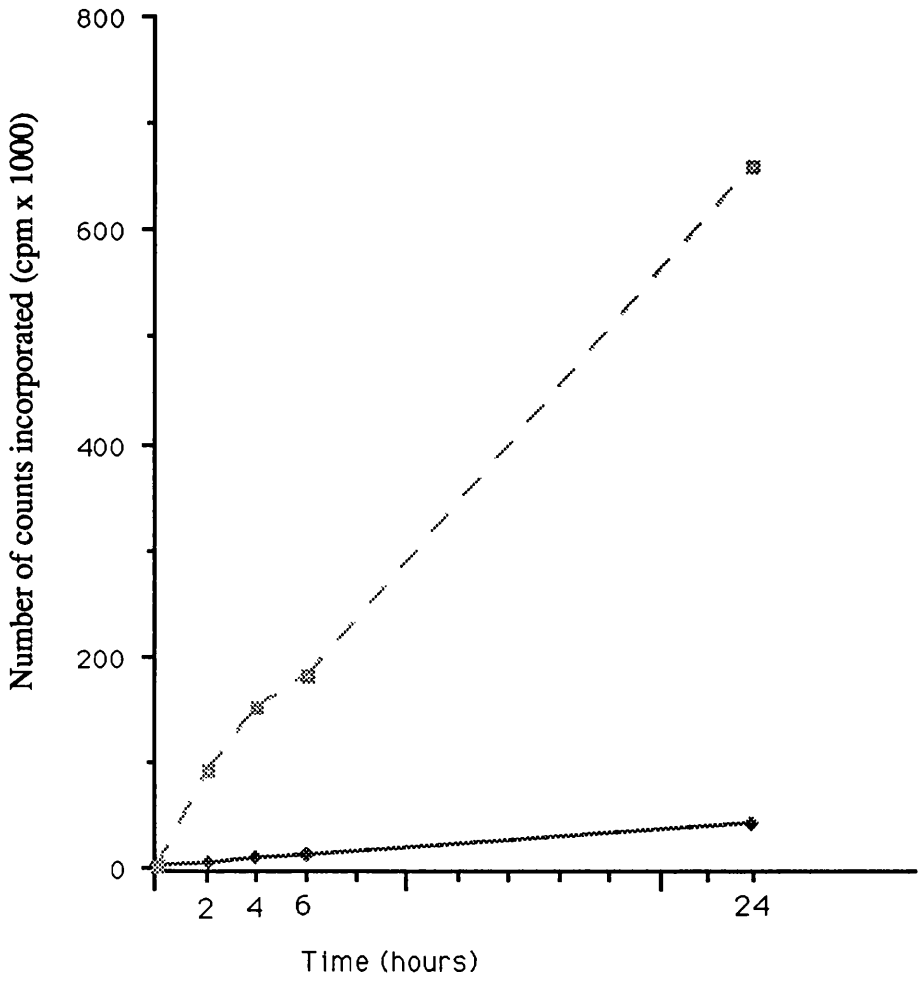
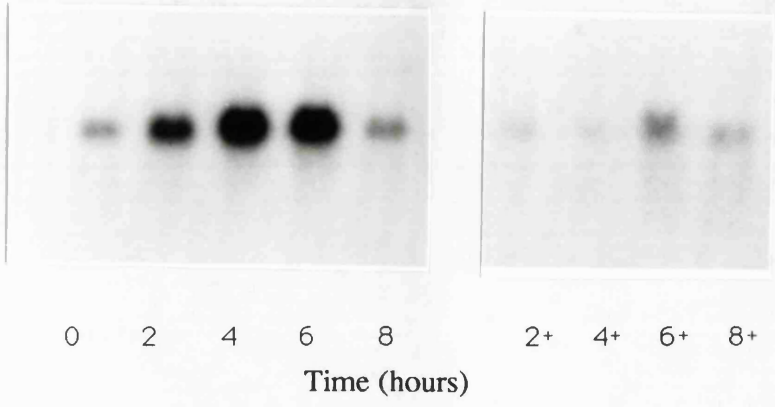


Figure 5.4 : The level of inhibition of translation by 10µg/ml cycloheximide, as measured by incorporation of ^{35}S methionine.

— — — Control

——— Cycloheximide



tPA mRNA

+ = Cycloheximide

Graph of standardized tPA mRNA levels.

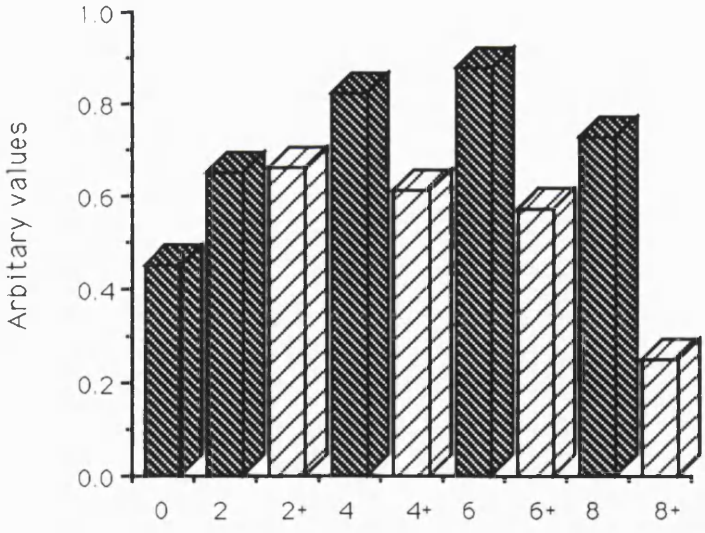


Figure 5.5 : The stability of tPA mRNA in A15A5 after inhibition of translation in the presence of serum.

Control Cycloheximide (10µg/ml)

ug/ml cycloheximide in the presence of serum. Figure 5.5 shows the effect of translation inhibition on tPA mRNA levels. The results show that tPA message remains stable and relatively high during the initial 6 hours before declining substantially by 8 hours. The general trend towards a gradual decrease in levels suggest that translation inhibition over a long period of time may increase the degradation of the tPA message.

5.6.3 The cellular localisation and the effect of actinomycin D and cycloheximide on the nuclear mRNA levels of tPA.

The results so far suggest that the tPA mRNA is stable over several hours in the A15A5 cell line. The mRNA stability in the ARBO C9 cell line could not be determined due to the considerably low basal level of the message. Therefore these data and the results from chapter 4 suggests that the constitutive higher level of the message found in the glioma cell line is primarily due to the increased stability of the mRNA in A15A5. To determine whether this is a nuclear or a cytoplasmic phenomenon, the distribution of the message in the nucleus and the cytoplasm of A15A5 cells was determined.

Events that can influence nuclear message levels include RNA processing, nuclear degradation and nuclear export of the mature mRNA. There are only a few reported cases of nuclear regulation of mRNA stability although PABP is known to traverse the nuclear membrane and also an ARE binding

protein has been isolated that is mostly in the nucleus.

Nuclear RNA was prepared from nuclei isolated from both A15A5 and ARBO C9 cells grown in the presence of serum. The procedure of isolation was identical to that used to isolate nuclei for nuclear run-on assays (chapter 3). The nuclei were given an extra wash to ensure there is no contamination of the nuclear preparations with cytosolic fragments. Cytoplasmic RNA was isolated according to Sambrook et al. (1989), (Methods and Material).

The cellular distribution of tPA mRNA in the A15A5 and ARBO C9 cell lines are presented in figure 5.6. The RNA was isolated from cells 24 hours after change of medium. The results show that the tPA transcript is predominantly confined to the nucleus of the A15A5 cells. The figure 5.6 also shows the actin message levels. Although the cytoplasmic actin levels were slightly lower, the tPA level when standardised to actin show that it is substantially higher in the nucleus than the cytoplasm.

In the ARBO C9, the cytoplasmic level of tPA was also found to be very low. The nuclear mRNA levels in ARBO C9 could not be ascertained in this experiment as the quantity of RNA used in the experiment was found to be too low (see actin level). This may have been due to leakage of the RNA from the sample well of the gel. However, nuclear level in the ARBO C9 cells was remeasured (figure. 5.7a) and was found to be low and comparable to cytoplasmic levels. These findings further

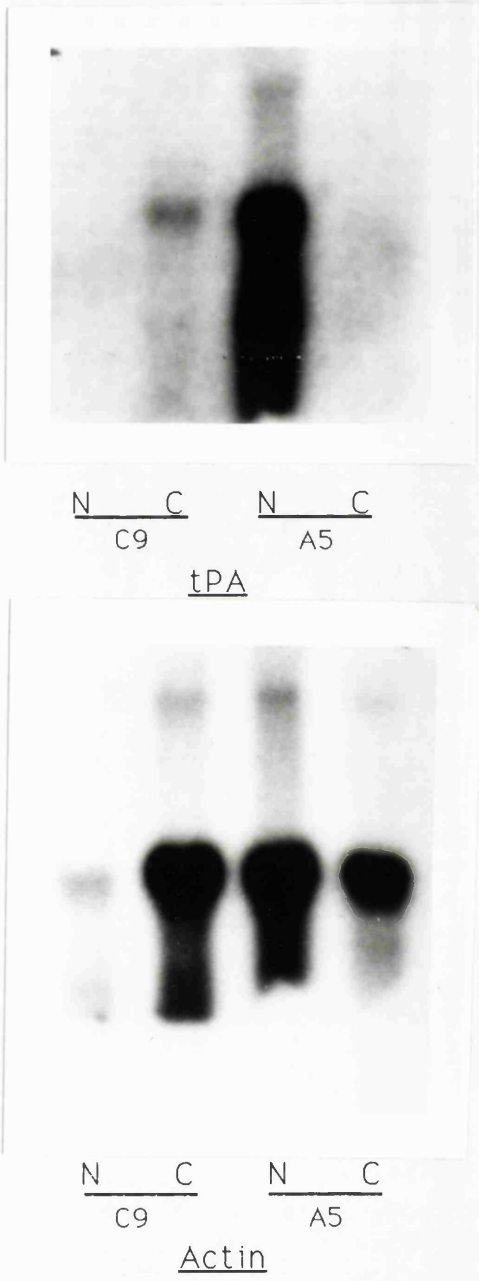


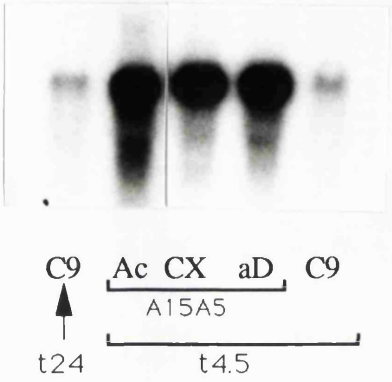
Figure 5.6 : The nuclear and the cytoplasmic distribution of tPA mRNA in A15A5 and ARBO C9.
N= nuclear C= cytoplasmic
A5= A15A5 C9= ARBO C9

substantiate results found with total mRNA levels; that is, the basal level of the tPA message is much lower in the the ARBO C9 cell line than in the A15A5.

The time course studies on total RNA indicated that tPA mRNA is relatively stable between 2 to 6 hours after transcription inhibition, whilst the normal levels increase rapidly between 2 to 6 hours (figure 5.3). To determine whether a similar effect can be seen in the nucleus, the RNA levels were measured at 4.5 hours (an intermediate time between 2 - 6 hours) in the nucleus after both transcription and translation inhibition.

The results presented in figures 5.7a and 5.7b shows that nuclear RNA level of tPA remain identical to basal level in the presence of both cycloheximide and actinomycin D. Therefore the difference between the basal levels and the actinomycin D treated total RNA levels at 4 hours may be due to increased degradation of the message in the cytoplasm.

The figure 5.7a also show the nuclear tPA mRNA level in the ARBO C9 cells at 4.5 hours. It was found to be approximately 10 fold lower compared to the nuclear levels of the message in A15A5 cells.



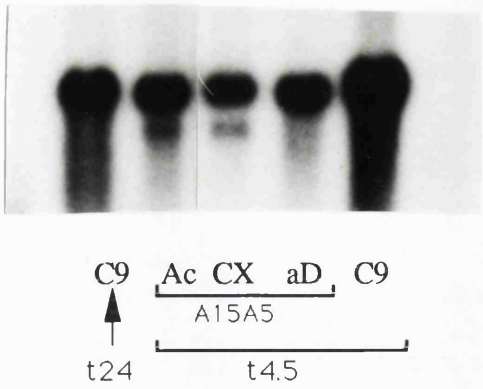


Figure 5.7a : The nuclear tPA mRNA levels at 4.5 hours in A15A5 and ARBO C9.

C9= ARBO C9; Ac= A15A5 control; CX= Cycloheximide; aD= actinomycin D; t = time (hours).

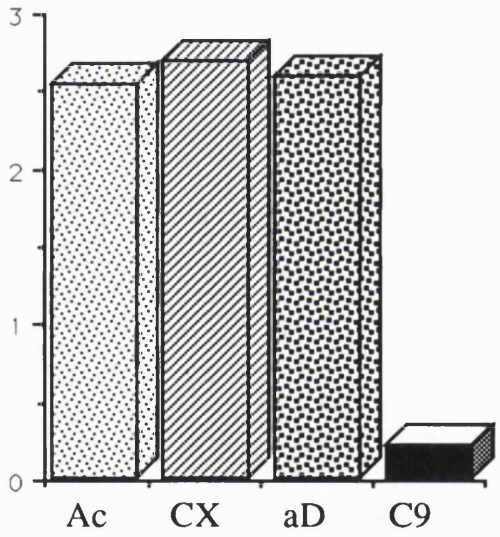


Figure 5.7b : The standardised results of nuclear tPA mRNA levels at 4.5 hours

5.7 DISCUSSION

The steady state stability of tPA mRNA in A15A5 glioma cells was measured after both transcription and translation inhibition. The concentration of actinomycin D used to inhibit mRNA transcription in these cells was 1ug/ml. This is lower than the more generally used concentration of 5ug/ml. Concentrations above 2.5 ug/ml were found to be toxic to these cells causing morphological changes and detachment of the cells from the substratum. The concentration of actinomycin D used in this study was sufficient to inhibit incorporation of ^{3}H uridine into the cells by greater than 95% in 4 hours and the cells remain viable over 24 hours.

The time course of tPA mRNA showed that the basal levels increase over the 6 hour period observed in the presence of serum. Results from other time courses in this study and this time course suggest that in the presence of serum mRNA levels of tPA reaches a peak between 6 to 8 hours and then declines slightly by 24 hours (figure 5.2c).

After transcription inhibition in the presence of serum the tPA mRNA levels were found to be stable up to 6 hours. As discussed earlier the tPA mRNA level in ARBO C9 is approximately 10 - 20 fold lower compared to A15A5 which indicate that the transcript is unstable in ARBO C9 cells, and nuclear run-on indicated that transcription does not account for this difference in the mRNA level. Therefore it can be concluded

that the increased tPA mRNA in A15A5 is probably due to increased stability of the message in the glioma cells .

The mRNA level in the absence of serum which represent behaviour of the message during collection of conditioned medium for PA activity assays show that tPA mRNA is expressed in the A15A5 cells although with a lag in time. It was noticed in several of the time courses, that change of medium leads to a transient degradation of the message irrespective of whether serum is present or absent. The course of this loss is unknown, it is possible it is due to the shock of altered conditions during the changing process itself, such as the drop in temperature and the CO_2 level when the cell are removed from the incubator. Subsequently the message level recovers as new message is transcribed, but the rate depends on the presence or absence of serum. In the presence of serum the peak level is reached much faster (6 to 8 hours) than in the absence of serum (>8 hours). However other work in this study showed that the total levels at 24 hours are comparable under both presence and absence of serum (figure 6.1).

When serum is absent from the medium there may be also increased degradation of the tPA message, as transcription inhibition leads to a gradual decline of the mRNA level over 6 hours. It is possible a serum factor or a factor induced by serum may be protecting the message from degradation in the A15A5 cells in the presence of serum.

One possible candidate is a factor similar to the poly-A binding protein. Studies by Berger et al. (1992) indicated that expression of PABP could be regulated by growth conditions. PABP is a translationally regulated polypeptide shown to be induced by serum. Several workers have shown that PABP has the ability to bind to poly A tails of mRNA and possibly suppress poly-A removal which generally leads to message degradation. Therefore it can be argued that in the absence of serum there is a suppression of translation of PABP or another mRNA regulatory factor that leads to the degradation of tPA mRNA in the absence of serum.

Cycloheximide attenuates translation by suspending transcripts on the polysomes, thus preventing synthesis of new proteins. Inhibition of translation in A15A5 does not lead to an immediate change in the levels of tPA message in these cells. The message levels remain stable up to 6 hours before undergoing a substantial decline by 8 hours. The reason for the decline is unclear. Cycloheximide mediated inhibition of translation may suppress synthesis of TFs required for transcription of tPA, therefore the decline may be a transcriptional effect. Translation inhibition may also inhibit proteins that maintain the stability of the tPA mRNA. Another theory that has been proposed for the mechanism involved in cycloheximide mediated increase in mRNA stability involves the action of cycloheximide itself. As cycloheximide acts by "freezing" ribosomes on mRNA forming stable complexes, it may make the transcripts inaccessible to RNases for degradation.

Cycloheximide has been demonstrated to show a multitude of effects on tPA message regulation in other cells. FSH induced tPA mRNA in PMSG primed rat granulosa cells was stabilised when cycloheximide is added 4 hours after stimulation by FSH (O' Conell et al., 1987). This effect can only be seen 12 hours after addition of cycloheximide. Furthermore if cycloheximide is added 30 mins before addition of FSH or LH it can be found to decrease the level induction by the hormones. Translation inhibition itself had no effect on the basal level of tPA mRNA in the absence of the hormone.

Another important observation in this study is the elevated levels of tPA RNA in the nucleus compared to the cytoplasm of the A15A5 cells. There are only a very few examples of increased stability of nuclear mRNA levels. Henderson et al (1992) has reported increased nuclear stabilization of uPA RNA in DMBA induced rat mammary adenocarcinomas. Others include dexamethasone induced nuclear stability of α1- acid glycoprotein mRNA (Vannice et al., 1984) and an increase in dihydrofolate reductase stability upon growth stimulation of a mouse sarcoma cell line (Leys et al., 1984).

The nuclear level of tPA mRNA in the ARBO C9 cells was approximately 10 fold lower. Therefore it can be concluded that the difference in the tPA message level between the two cell lines is primarily due to the increased stability of the message in

the nucleus of the glioma cells.

Also in the nucleus of A15A5, the RNA levels at 4.5 hours after inhibition of both transcription and translation remain comparable to untreated levels whilst in total mRNA, inhibition causes a slightly lower level compared to untreated cells. Which suggest degradation of the message takes place primarily in the cytoplasm.

Several of the genes whose messages are stabilized by translation inhibition have AU-rich elements (ARE) or instability elements in their 3' UTR. Several proteins are known to bind to these elements and target their destruction. It is proposed that translation arrest leads to suppression of ARE binding protein levels thus inhibiting degradation of these messages. The tPA mRNA has two AUUUA pentamers in the 3' UTR within a region of high AU bases.

So what accounts for the possible differences in degradation of the tPA message in the nucleus and the cytoplasm? It was discussed in the introduction that several ARE-binding proteins are present in the nucleus and have homology to hnRNPs involved in nuclear RNA processing, but it is not known whether hnRNPs or trans-acting ARE binding proteins have a role in the degradation of nuclear mRNA. Bohjanen et al. (1991) analysed binding capacity of two ARE-binding proteins AU-A and AU-B which bind to the 3'UTR ARE of GM-CSF in T-cells. AU-A was primarily in the nucleus and AU-B in the cytoplasm. AU-A was

a general ARE binding factor that could bind to both lymphokine and protooncogene (c-myc) AREs, while AU-B had greater affinity and specificity to GM-CSF 3'ARE and competed with AU-A for binding to the ARE. T-cell receptor mediated stimulation which leads to an increase in instability of lymphokine mRNA was found to induce AU-B levels. However co-stimulation with PMA which increases the stability of GM-CSF message was found to cause a decrease in binding activity of AU-B.

These results indicate that the predominantly cytoplasmic protein AU-B is responsible for the increase in the degradation of the GM-CSF mRNA in stimulated T-cells. Although the nuclear factor (AU-A) bound to the 3'UTR ARE it was proposed to have no function in the regulation of stability of GM-CSF. It is possible that AU-A maybe a hnRNP involved in nuclear processing of the transcripts. These results also imply that AU-binding proteins can discriminate between different AU elements, as AU-A can bind to both c-myc and GM-CSF, while AU-B cannot bind to c-myc.

A similar model could be proposed for the altered regulation of tPA message in the two cell lines. The low levels of the message in the nucleus and in the total mRNA of ARBO C9 suggest that the message is unstable in both compartments of the ARBO C9 cells. Therefore it can be proposed that in ARBO C9 the message may be selectively targeted for rapid destruction in the nucleus and possibly also in the cytoplasm after its immediate transport to the cytoplasm. While in A15A5 the factors that target

degradation of the tPA transcript in the nucleus may be suppressed or inactivated leading to the accumulation of the message in the nucleus.

It is also possible that nuclear / cytoplasmic transport may have a role in this process. But there is no evidence to suggest that nuclear export of tPA mRNA is impeded in A15A5 cells, because the PA protein is expressed at high levels in A15A5 which implies continuous export of the mRNA to the cytoplasm for translation.

Therefore from these data it could be suggested that differential expression of tPA mRNA in the glioma and the normal cell line is primarily due to the increased stability of the tPA message in the nucleus of A15A5 cell line. It is possible that there is an alteration in the 3'ARE binding proteins in the nuclei of the cell line. These factors could be those that target for increased destruction of the transcripts or those that protect mRNA from nucleases.

The absence of or the very low levels of the tPA mRNA in the cytoplasm of these cells suggest that the message undergo degradation in the cytoplasm.

CHAPTER 6: Regulation of tPA mRNA in A15A5 by modulators of intracellular signalling

6.1 INTRODUCTION.

The previous three chapters were devoted to the investigation of mechanisms responsible for the differential regulation of the tPA message in A15A5 and ARBO C9 cell lines. However the following two chapters will concentrate on the modulation of the tPA mRNA in A15A5 cells by three extracellular agents known to influence the fibrinolytic activity of the cells.

One of the forms that living organisms employ to regulate tissue or cell specific gene expression is through the use of extracellular agents that can modify rates of transcription of these genes. Several different extrinsic agents including hormones, growth factors and morphogens are involved in this process. The signals from these agents are transduced to their affected genes through a cascade of intracellular signal molecules leading to changes in the spectrum and the expression of the cellular genes.

The regulation of transcription through intracellular signalling involve activation of specific transcription factors in the nucleus. These factors then bind to the regulatory sequences in the promoters to modify transcription either positively or

negatively (discussed in the chapter 3). This activation which involves either phosphorylation or dephosphorylation of the transcription factors does not require new protein synthesis.

Several of the agents that induce intracellular signal transduction including cholera toxin (CT), epidermal growth factor (EGF) and the glucocorticoid analogue dexamethasone (Dex) have been shown to influence both the activity and the RNA levels of tPA in several cell lines.

6.2 Regulation of mRNA levels by cholera toxin.

Cholera toxin with forskolin belongs to the group of agents that modulate intracellular cAMP levels. CT causes a persistent activation of cellular adenyl cyclase leading to a permanent increase in intracellular cAMP levels which in turn activate cAMP dependent protein kinase A (pk-A). This is a phosphoprotein composed of two modules. Once activated the components dissociate and the module containing the kinase activity migrates into the nucleus and phosphorylates its effector protein.

CT has been shown to elevate tPA mediated fibrinolytic activity of both lysate and conditioned medium (CM) from the A15A5 and the ARBO C9 cell lines (Neame and Roscoe, 1988). The lysate activity represents membrane associated tPA whilst CM represents secreted or accumulated protein over 24 hours. CT was found to cause an approximately 2-fold increase in the fibrinolytic activity of conditioned medium from A15A5.

Furthermore forskolin which also activate intracellular cAMP can induce PA activity in A15A5 cells.

Elevation in the cAMP levels through inducers of intracellular cAMP have been demonstrated to increase PA activity and PA mRNA in several other cell lines. Administration of CPT-cAMP an analogue of cAMP to primary rat hepatocyte cultures for 16 hours was found to cause a 4 fold rise in tPA mRNA (Heaton et al., 1989), while in granulosa cells there was a 20-fold elevation in the tPA message by cAMP (Ohlsson et al., 1993). Furthermore calcitonin, another inducer of cAMP has been shown to induce uPA dependent PA activity in LLC-PK porcine kidney cells.

6.3 Regulation of PA activity by epidermal growth factor.

Another extrinsic agent that elevate PA activity in the A15A5 cell line is the polypeptide growth factor EGF (epidermal growth factor). The effect of EGF mediated rise in fibrinolysis is primarily seen in the CM of A15A5 (Neame and Roscoe, 1988).

There are several families of growth factors including Fibroblast growth factor (FGF) family, platelet-derived growth factor family (PDGF), hepatocyte growth factor (HGF) family and the EGF family. Other members of the human EGF family include TGFα and amphiregulin. Growth factors activate intracellular signalling through binding to their respective receptors on the cell surface. Some receptors such as the receptor for EGF (EGFR), which it shares with TGFα have

intrinsic protein kinase activity. Amphiregulin also binds to the EGF receptor but with a lower affinity (Todaro et al., 1980). Therefore these receptors can directly phosphorylate and activate intracellular signalling molecules.

EGF has been demonstrated to mediate phosphorylation of both phospholipase C (Margolis et al., 1989) and casein kinase II (Ackerman and Osheroff, 1989). Phospholipase C phosphorylation leads to the activation of the intracellular second messengers inositol triphosphate (IP3) and diacylglecerol (DAG). IP3 causes the release of Ca^{2+} from intracellular stores leading to the activation of Ca^{2+} / calmodulin dependent protein kinases (Cam kinase) whilst DAG result in the activation of protein kinase C. Therefore stimulation of EGFR by EGF leads to the induction of tyrosine as well as serine and threonine kinases.

One of the principal effect of growth factor stimulation of cells is the activation of immediate-early genes. These genes code for several known or probable transcription factors (TFs) including c-myc, c-jun and c-fos. Fos and Jun proteins are two of the most well studied TFs. These two proteins dimerize either as Jun-Jun or Jun-Fos to form the complex AP1 which bind to the AP1 (activator protein 1) or TRE (TPA response element) sites on promoters to regulate gene transcription.

EGF is also known to stimulate PA activity in other cells or cell

lines . Lee and Weinstein (1978) demonstrated increased PA activity in HeLa cells after treatment with EGF. However they did not measure the type of PA responsible for this activity. EGF was also found to induce both uPA and tPA activity in lung fibroblasts (Laiho et al., 1986) and tPA in rat granulosa cells. In human microvascular endothelial cells EGF leads to an elevation in both tPA activity and migration of the cells.

While CT and EGF were demonstrated to augment the high PA activity of A15A5 cells, the glucocorticoid dexamethasone causes a considerable suppression of this activity.

6.4 The regulation of PA activity by dexamethasone.

The steroid hormone superfamily to which dexamethasone belong to, also include thyroid hormones, retinoic acid and vitamin D. These hormones can influence gene transcription directly through binding to their intracellular receptors. The receptors acts as transcription factors by binding to hormone response elements in the promoters. The activation of the hormone receptors takes place through their phosphorylation. Nielsen et al., (1977) has demonstrated that suppression of phosphorylation or dephosphorylation can inactivate glucocorticoid receptor activity in L-cells.

The cellular location of the receptors varies for different hormones. The glucocorticoid and mineralocorticoid receptors are found predominantly in the cytoplasm. While the receptors for the other members of the group generally exist within the

nuclear envelope.

Previous studies demonstrated that the glucocorticoid dexamethasone causes a 5 fold suppression of the high PA activity of CM from A15A5 cells. It also more or less eliminate PA activity of lysates from these cells.

Others have demonstrated dexamethasone to have an opposing effect on tPA activity and its mRNA. Heaton et al. (1989) found dexamethasone to cause a 65% decrease in tPA mRNA levels in primary rat hepatocytes. Meanwhile in HTC rat hepatoma cells there was a dexamethasone mediated 90% reduction in fibrinolytic activity in the membrane fraction of the cells. However, in these cells there was also a small but simultaneous increase in tPA antigen levels. Dexamethasone was also found to induce transcription and mRNA levels of tPA in HT 1080 human fibrosarcoma cells (Medcalf et al., 1988).

In this chapter the effect of the three extracellular agents EGF, CT and dexamethasone on the expression of tPA mRNA in A15A5 is investigated.

6.5 Results.

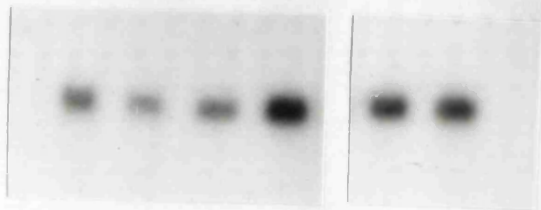
6.5.1 The modulation of tPA mRNA levels in A15A5 by cholera toxin.

As mentioned earlier CT is known to induce PA activity in both cell lysates and CM from glioma cells. The figure 6.1 shows the level of tPA mRNA 24 hours after addition of CT to the growth medium. These levels were measured under normal growth conditions (15% FCS) and in serum-free medium. In chapter 5 tPA was shown to be expressed under both presence and absence of serum, although the kinetics of expression was different.

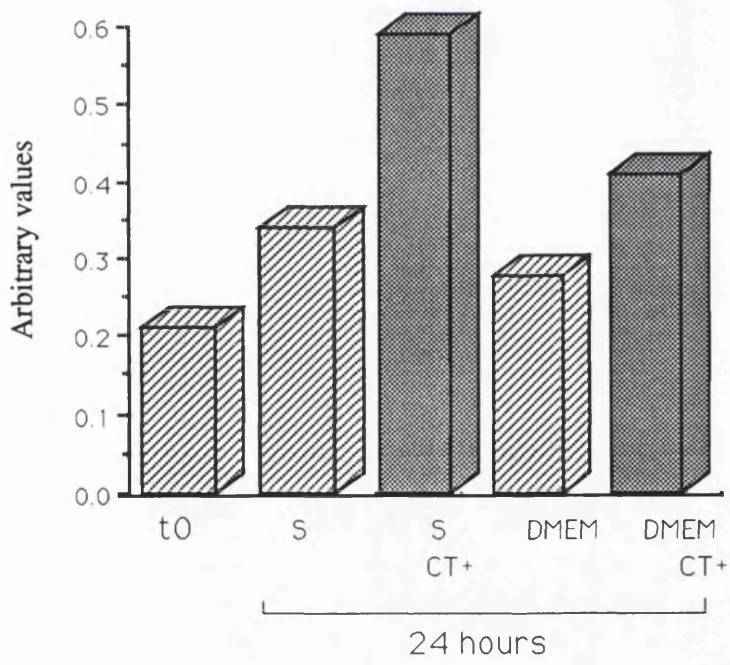
The normal tPA mRNA levels show (figure 6.1) only a small reduction in the message after 24 hours in the absence of serum. Which indicate that over 24 hours the lack of serum does not significantly inhibit the expression of tPA mRNA in these cells.

Administration of CT to the growth medium was found to elevate tPA message under both conditions. However the induced message levels were higher in the presence of serum than in the absence of serum. In the presence of serum CT induced tPA mRNA by approximately 2 fold compared to untreated controls, while in the absence of serum the difference was less.

A time course of tPA mRNA induction in the absence of serum



0 24 S S CT+ D D CT+



to = mRNA at change of medium ; S = 15% serum ; D= DMEM (no serum) ; CT = cholera toxin; 24 = unchanged medium at 24 hours-not shown

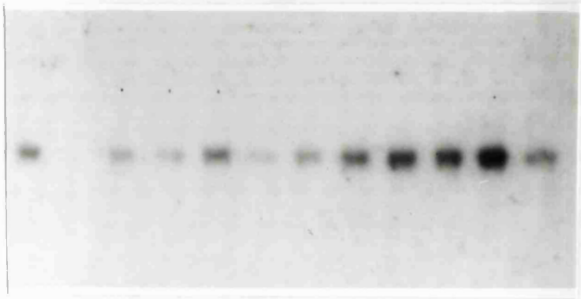
Figure 6.1 -Induction of tPA mRNA at 24 hours by cholera toxin in different concentrations of serum

by CT (figure 6.2) shows a time dependent rise in the message levels. The induced levels were higher than the comparable normal level at all time points except at 2.5 hours. The reason for this low levels at 2.5 hours is unclear. It is possible that CT transiently elevate a suppressor of tPA transcription or an inducer of tPA message degradation during this period.

As CT mediated signal transduction directly influence transcription of genes, nuclear run-on transcription assays were carried out to determine the effect of CT on the transcription of the tPA message. Figure 6.3 shows the level of transcription 5 hours after treatment with CT. Densitometric analysis indicate an approximate 2 fold rise in tPA transcription in the presence of CT. These signals were normalized to rat repetitive signals.

6.5.2 The effect of EGF on the expression of tPA mRNA in A15A5 cells.

Figure 6.2 also shows the effect of EGF on tPA mRNA in the absence of serum. The time course shows that in the presence of EGF the tPA mRNA levels reaches a peak at 7.5 hours and declines by 24 hours. It is only at this time point that EGF induced tPA mRNA level rises above the control mRNA level during the time course. Which suggest that EGF mediated induction of tPA may be transient. During the rest of the time there is a small inhibition of the message level, which could be due to either reduced transcription of enhanced degradation of the message.



D C E D C E D C E D C E

2.5 5.0 7.5 24

TIME / HOURS

D= DMEM, C = cholera toxin, E = EGF

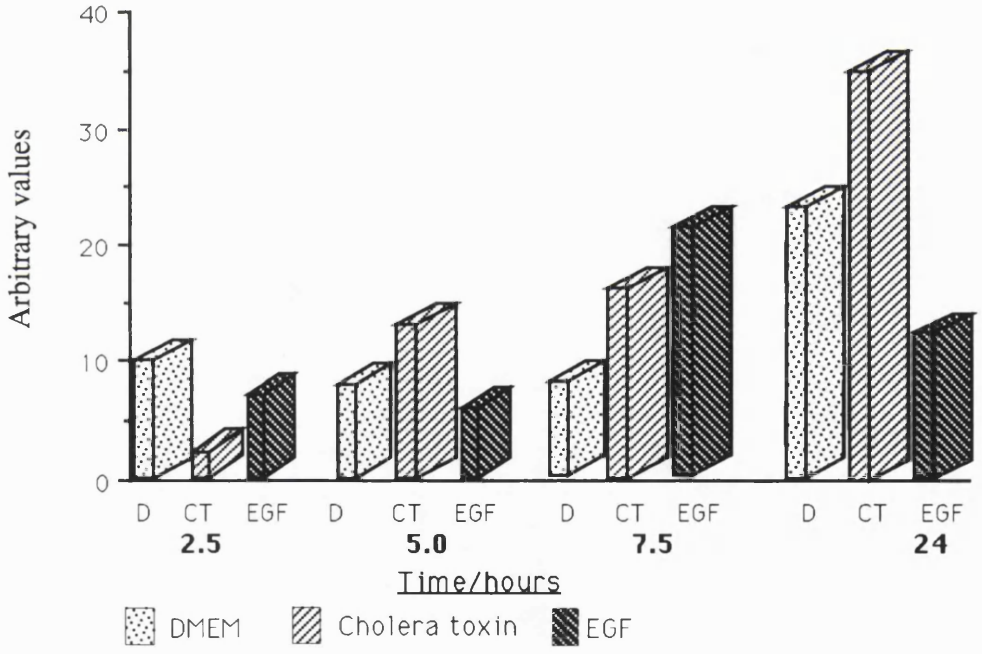


Figure 6.2- The time course of modulation of tPA mRNA in A15A5 by CT and EGF in the absence of serum.

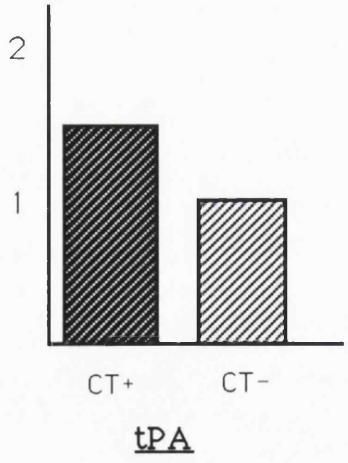


Figure 6.3 : Nuclear run-on of the effect of cholera toxin on tPA mRNA transcription in A15A5.

6.5.3 The effect of dexamethasone on the expression of tPA mRNA.

The cells were treated with 10^{-7} dexamethasone and a time course of its effect on tPA mRNA was determined. This concentration of dexamethasone was found to considerably suppress PA activity in conditioned medium from A15A5 cells in previous studies.

The figure 6.4 shows the effect of dexamethasone on the expression of tPA mRNA in the absence of serum. During the initial 6 hours tPA mRNA levels in the presence of dexamethasone remain comparable to basal message levels. At 8 hours there is a small elevation due to dexamethasone, which however, diminishes substantially by 24 hours.

The kinetics of induction of basal tPA transcript in the absence of serum was found to be identical to the earlier result (depicted on figure 5.3). These results confirm that there is a time lag in the synthesis of tPA messages in A15A5 cells when the growth medium is replaced with medium lacking serum .

The time course indicate that although dexamethasone causes the PA activity of conditioned medium from A15A5 cells to be almost abolished it is not due to a direct effect on the tPA mRNA itself.

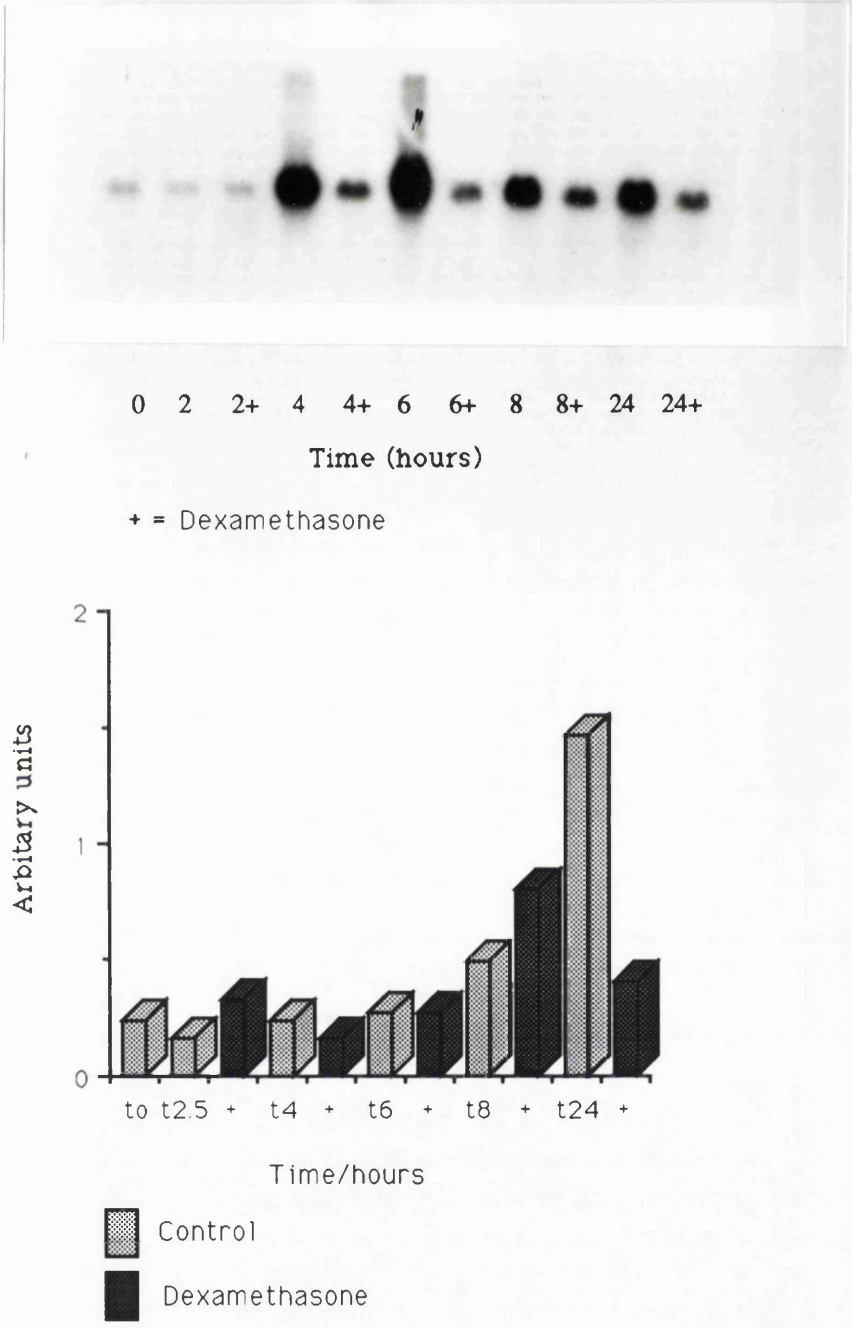


Figure 6.4 - The effect of dexamethasone on tPA mRNA in the absence of serum

6.6 DISCUSSION

The influence of three extrinsic modulators of intracellular signalling on the expression of tPA mRNA in the A15A5 cells line was investigated. The three agents studied CT, EGF and dexamethasone have been previously shown to effect the PA activity of this cell line.

Studies on the steady state level of the tPA RNA 24 hours after treatment with CT revealed that the message could be induced by CT in the presence and in the absence of serum. The lower level of the message induced by CT in the absence of serum may be due to a post-transcriptional affect on the tPA mRNA. It was shown earlier (chapter 5) that the tPA transcript is less stable in the absence of serum.

The CT mediated induction of tPA mRNA in the presence of serum was approximately 2-fold. The nuclear run on assay was used to confirm that transcription is primarily responsible for this induction of the total message levels by CT.

These results substantiate previous studies on the effect of CT on PA activity, which showed that CT causes an elevation of tPA mediated fibrinolysis in both CM and cell lysates of the A15A5 cell line. The fibrinolytic activity of the CM was elevated by approximately 2-fold, and the cell lysate activity to a lesser extent.

The time course study indicated a continuous induction of tPA message by CT. These results reflect the mechanism of action of cholera toxin on intracellular signalling. CT causes a persistent activation of adenyl cyclase leading to continuous production of intracellular cAMP. Stimulation of cAMP-dependant protein kinase A by cAMP leads to the phosphorylation of the nuclear transcription factor CREB, which can initiate gene transcription through binding to CRE elements in promoters. Direct phosphorylation of CREB leads to transcription has been demonstrated by Gonzales and Montiminy (1989). Many genes that respond to elevated intracellular cAMP have a CRE element with the sequence GACGTCA within their promoters. This element is present in the rat tPA promoter, therefore it can be proposed that cAMP mediated elevation in transcription and the continuous rise in tPA mRNA seen in the time course is due to the activation of the CRE in the tPA promoter.

The human and the mouse promoters do not have a CRE but has the closely related TRE which can also respond to intracellular cAMP.

The polypeptide growth factor EGF induced tPA in a time dependent manner. In this time course maximal tPA RNA levels were reached at 7.5 hours, which then falls by 24 hours. In the presence of EGF the tPA levels seem to be suppressed compared to the controls except at 7.5 hours. This suggest that EGF may be also inducing possible proteins that targets

degradation of the tPA mRNA.

Previous studies have established that treatment of EGF for 24 hours leads to an elevation of PA activity in CM of A15A5, without a significant rise activity in the cell lysates. The activity in CM represents accumulated protein, or the mRNA transcribed earlier in time. The lysate activity represent cell associated PA and may represent more immediate levels of tPA message. As can be seen in figure 6.2 the mRNA level of tPA declines by 24 hours and may relate to the cell associated protein level, whilst PA activity in the CM may reflect mRNA levels earlier in the time course (ie 7.5 hours).

EGF is known to induce tPA mRNA and activity in other cell lines. In human microvascular endothelial cells, EGF causes a time dependent (Mawatari et al., 1991) elevation of tPA message. There was a 2 to 3 fold rise in mRNA levels at 9 hours and high protein levels between 6-12 hours which decline to basal levels by 24 hours. Here again there is an EGF related early rise in mRNA as found in the A15A5 cell line.

Dubeau (1988) found EGF induces tPA mRNA in a non-neoplastic human urothelium cell line, but not in a neoplastic urothelium cell line that did not produce tPA initially. These results suggest that action of EGF may be cell specific.

The expression of EGF receptor is also an important consideration for cellular response to EGF. The EGFR is

overexpressed in several tumours including human brain (Lieberman et al., 1985), breast, bladder and lung tumours.

Studies by Wang et al. (1989) demonstrated that labelled EGF binds predominantly to extracts of glial cells from primary cultures from neonatal rat brain than to extracts from neuronal cultures. EGF was also found to stimulate incorporation of ^{35}S methionine into both cellular and secreted proteins from cultured glial cells, and furthermore induced a dose dependent incorporation of ^{3}H uridine in to these cells indicating induction of both transcription and translation in the glial cells by EGF. This data suggest that the glial cells have the ability to respond to EGF probably through elevated expression of EGFR on their cell surface.

Additionally, ENU has been shown to cause mutations in the neu oncogene which codes for an analog of EGFR, in schwannomas but not in other brain tumours (Nikitin et al., 1991).

EGF mediates its action primarily through activation of intracellular phospholipase C through binding to its receptor EGFR which has intrinsic TK activity. Phospholipase C initiate intracellular 2nd messengers that activate both Cam kinase and protein kinase C. EGF has also been shown to activate casein kinase II (Ackerman and Osheroff 1989, Ackerman et al., 1990), an ubiquitous cyclic nucleotide dependent protein

kinase with the ability to phosphorylate the cAMP response element binding protein.

Although the tPA mRNA possess a CRE in its promoter, whether EGF mediated elevation of the message in A15A5 is due to the direct induction of transcription of tPA or to a secondary post-transcriptional event is yet to be elucidated.

The glucocorticoid dexamethasone is a known repressor of the high fibrinolytic activity of A15A5 cells. Previous work has shown that the glucocorticoid dexamethasone causes a suppression of PA activity in both cell lysates and CM from A15A5 cells. The time course of the effect of dexamethasone on mRNA levels found that dexamethasone has little influence on the tPA message over the initial 8 hours.

Subsequently there is a decline in the message level compared to the basal level, however this is not as great as the 5-fold suppression in PA activity caused by dexamethasone. Therefore the results indicate that the primary effect of dexamethasone on fibrinolytic activity of A15A5 cells is not due to an effect on tPA mRNA.

Other workers have also demonstrated an opposing effect of dexamethasone on PA activity and mRNA. In HTC rat hepatoma cell line dexamethasone while inhibiting PA activity, induced tPA mRNA 2 to 3 fold between 4 and 6 hours during a time course study (Heaton and Gelehrter, 1989). Medcalf et al.

(1988) showed that dexamethasone elevates both cell associated and secreted tPA protein assayed over 48 hours. The tPA mRNA levels were also increased at 8 hr in this study, despite the down regulation of PA activity.

The effect of dexamethasone on tPA is paradoxical. Whilst having little effect on tPA mRNA levels, dexamethasone strongly inhibit PA activity of A15A5 glioma cells. Others have found that dexamethasone modulates PA activity of cells primarily due to the induction of PAI-I, a specific inhibitor of tPA. The influence of dexamethasone on the expression of PAI-1 mRNA in A15A5 was also investigated and is reported in chapter 7.

CHAPTER 7 - REGULATION OF PLASMINOGEN ACTIVATOR INHIBITOR-1 IN A15A5 & ARBO C9 CELL LINES

7.1 INTRODUCTION

Plasminogen activator inhibitor 1 (PAI-1) is the major physiological inhibitor of tPA in plasma (Chmielewska et al. 1983) and tissues. PAI-1 can react with both the single and two chain forms of tPA to form an enzymatically inactive complex. PAI-1 which acts as an alternative substrate to tPA is cleaved at an Arg_{346}-Met_{347} peptide bond releasing the carboxy terminal complex (reviews- Sprengers and Kluft, 1987, Andreasen et al., 1990).

PAI-1 belong to the serine protease family of inhibitors (SERPINS) which also include PAI-2 and protease nexin. The two PAIs were originally named endothelial type and placental type according to their sites of isolation. However, as both types were later found in several types of tissue they were termed PAI-1 and PAI-1-2 respectively. PAI-1 can inhibit not only tPA, but also uPA.

tPA has high affinity for fibrin and its activity is elevated when bound to fibrin, furthermore tPA is protected from inhibition by PAI-1 when bound to fibrin (Chmielewska et al., 1988). Many neoplastic cells are known induce fibrin deposition (Zacharski et al., 1986). This may protect cell bound tPA which is probably involved in tissue invasion at secondary sites from

becoming inactivated and also increase the proteolytic activity at the host-tumour interface.

PAI-1 is a glycoprotein with a molecular weight of 54000. In the ECM it is found bound to vitronectin; the bound form of PAI-1 has greater stability than the free form (Wiman et al., 1988). It has been also suggested that the bound form of PAI-1 may be arranged in a conformational form that present the Arg-Met bond for optimal interaction with tPA (Carrell et al., 1991). In cultured cells PAI-1 is secreted in an active form which is then rapidly converted to an inactive latent form. This latent form can be reactivated by denaturants such as SDS and guanidium chloride (Heckman and Loskutoff, 1985).

Although initially thought to be mainly endothelial specific many different types of cells synthesize PAI-1 including human hepatocytes in culture (Sprengers et al., 1985), several hepatoma cell lines (Coleman et al., 1982), granulosa cells (Ny et al., 1986), MJZJ melanoma cells (Wagner et al., 1986) as well as endothelial cells (Pannekoek et al., 1986).

As the fast acting principal regulator of tPA and thus plasmin, PAI-1 has a significant role in thrombolytic events under normal physiological conditions. Decreased plasma fibrinolysis due to increased expression of PAI-1 and not due to lowered tPA levels has been implicated as an important factor in thrombosis and coronary arterial disease (Paramo et al., 1985).

7.2. Modulation of PAI-1 by extrinsic agents.

A multitude of extrinsic agents have been shown to modulate PAI-1 mRNA and its activity in a variety of cells. These include bacterial endotoxin which induces both message and protein levels in mouse endothelial cells, tumour necrosis factor (TNF) which elevates PAI-1 activity and mRNA levels in human endothelial cells (van den Berg et al., 1988) and interleukin 1 (IL-1) and bacterial lipopolysacharide in cultured rat endothelial cells (Emeis and Kooistra, 1986). IL-1 also elevates PAI-1 antigen and mRNA in human endothelial cell cultures (Schleef et al., 1988). However, TNF was found to have no affect on PAI-1 in a number of human cell lines (Georg et al., 1989) and in primary cultures of human and rat hepatocytes (van Hinsberg et al., 1988).

These workers have shown that many of these extrinsic agents including dexamethasone regulate PAI-1 at the level of transcription. Other groups have suggested the possibility that PAI-1 may be regulated through stability, as both the rat and the human PAI-1 transcripts contain AU-rich elements in their 3' UTR. The rat PAI-1 contain 2 AUUUA pentamers within this region. In the human there are 2 forms of PAI-1 (Ny et al., 1986, Pannekoek et al., 1986), a 3.2 kb form which have 2 AREs and a 2.4 kb form which lacks one of the AU elements. Both forms are derived from same hnRNA through differential processing and polyadenylation (Bosma et al., 1988). Some workers found the 2.4 kb to be more stable (Medcalf et al.,

1988), whilst others showed that its the 3.2 kb PAI-1 that is more stable (van den Berg et al., 1988). Cytokines such as TNF and IL-1 are known to enhance stability of mRNAs with 3' AREs and both of these agents have been shown to increase the steady state level of 3.2 kb PAI-1 in humans. It is possible that this increase is due to an increase in stability, though it is also possible that the 3.2 kb form is preferentially processed in the nucleus.

S1 DNA digestion assays on the promoter of the rat PAI-1 identified 8 protein footprints (Johnson et al., 1992). Five of the footprints were shown to bind to known TFs. There were 3 sites for SPI like proteins, one CTF/NFI and one that overlapped a SPI and a CTF/NFI site. Definite binding proteins for 3 footprints could not be identified.

7.3. Expression of PAI-1 in tumour cells.

There is contrasting evidence for the expression of PAI-1 protein and mRNA in neoplastic cells. It would be obvious to expect suppression of PAI-1 activity in tumour cells as it would further enhance PA activity and thus increase the proteolytic properties of the cells.

In contrast to this expected behaviour there is evidence that some tumours express higher PAI-1 activity simultaneously with increased PA levels. Study by Quax et al. (1990) on 22 human tumour cell lines of various origins that expressed uPA, tPA or both, found that many of these cells also expressed the

main inhibitors of PA concurrently. 20 out of the 22 expressed tPA protein and 16 out of the 22 had either PAI-1 or PAI-2. More interestingly there was a correlation between expression of PAs and their inhibitors, that is the cells with the highest levels PA also generally produced highest levels of inhibitors.

In transplantable murine lewis lung carcinoma Kirstensen et.al. (1989) found that central areas of the tumours expressed elevated levels of both uPA and PAI-1, whilst the peripheral tissue which showed invasion and local tissue destruction expressed only uPA protein. It was suggested that PAI-1 within the tumour may act to prevent plasmin mediated destruction of the tumour's own structure.

Work by other groups have indicated that oncogenic transformation leads to suppression of PAI-1 activity. Cohen et al. (1989) discovered that oncogenic transformation of rat 1 fibroblast cells with V-ras or EJ-ras leads to a down regulation of the high PAI-1 levels found in these cells. However there was a modest but concomitant rise in PAI-2 mRNA in these cells.

Transformation of other cell lines with v-myc and v-src was also found to suppress PAI-1 levels, indicating that PAI-1 expression could be regulated by several oncogenes with different modes of action. That is myc is a nuclear factor, v-src is a non-receptor tyrosine kinase and ras is cytoplasmic GTpase.

7.4. The activity of PAI-1 in normal tissue.

A correlation between PA and its inhibitor is also seen in some of the rat tissues examined by Lucore et al. (1988) where it was found that those tissue expressing high levels of PA also contained elevated PAI-1 levels. These data suggest that in normal physiological events the total PA activity may be regulated through counteracting PA levels with PAI-1 activity thus confining PA activity to target sites and preventing uncontrolled proteolysis and tissue damage. Therefore it has been proposed that it is the imbalance in this regulation that lead to abnormal proteolytic activity such as those found during tissue invasion and metastasis of tumours. In those cells where both agents are elevated simultaneously the change in the total PA activity is probably due to disproportionate change in one protein compared to the other.

It was demonstrated in the earlier work that tPA mRNA is expressed at a higher level in the glioma cell line than in the normal cell line. This elevation was shown to be primarily due to the enhanced stability of the tPA mRNA in A15A5. However the difference in the message levels does not account for the even greater difference in the fibrinolytic activity found between the cell lines. Therefore it was decided to investigate the expression of PAI-1 mRNA in these cell lines to determine whether altered expression of PAI-1 could account for differences in the PA activity.

7.5. RESULTS

7.5.1 The transcriptional regulation of PAI-1 mRNA

The level of transcription of the PAI-1 mRNA in the two cell lines was measured to determine whether PAI-1 was transcribed at a lower rate in A15A5 than in ARBO C9.

The rate of transcription was measured using the nuclear run on assay as described in chapter 4. The nuclei were isolated from cells growing in 15% serum, and 4 hours after change of medium.

Figure 7.1 shows the level of transcription of PAI-1 in the A15A5 and the ARBO C9 cell lines. The signals were analysed densitometrically and standardised to rat repetitive sequences. The results indicated that PAI-1 is transcribed at an almost identical rate in A15A5 and in ARBO C9. The figure also show the rate of transcription of tPA compared to PAI-1. The constitutive transcription of PAI-1 was found to be lower than tPA in both cells which suggest that PAI-1 mRNA levels may be identical in both cell lines. However, it is possible that PAI-1 is also regulated at a post-transcription level as found with tPA in A15A5. That is, despite the comparable level of transcription of tPA between the cell lines the stability of the tPA mRNA in A15A5 is much higher

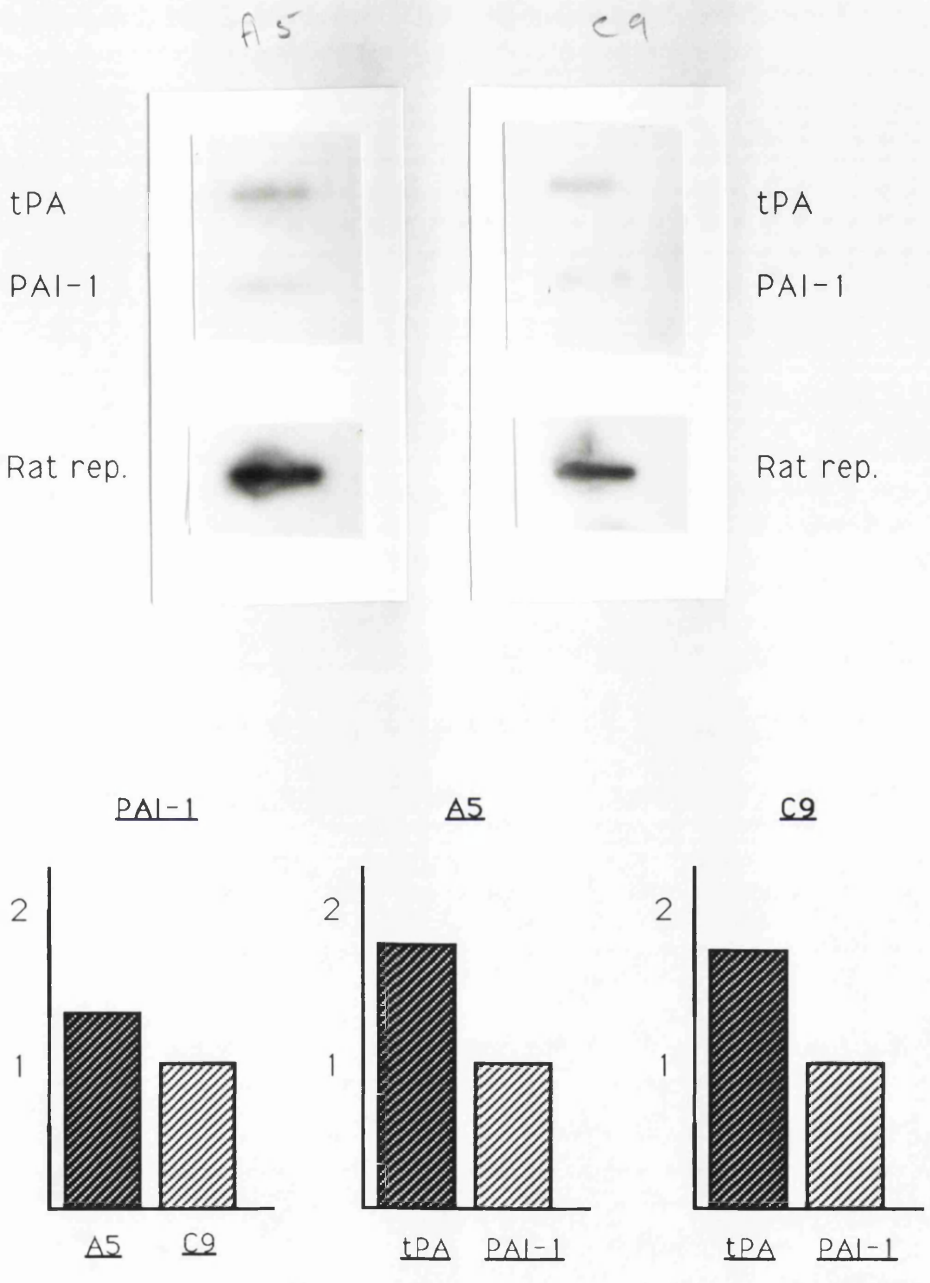


Figure 7.1 : The rate of transcription of PAI-1 in A15A5 and ARBO C9.

7.5.2 Post-transcriptional regulation of PAI-1 in A15A5 and ARBO C9.

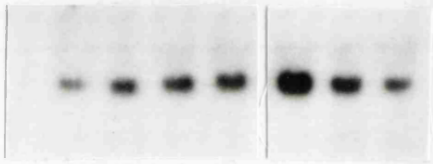
7.5.2a :The stability of PAI-1 in A15A5

Time course studies were carried out to investigate the expression of PAI-1 mRNA under normal growth conditions and to determine the stability of the PAI-1 message in A15A5.

The stability of the PAI-1 message was determined on total mRNA preparations. The transcription in A15A5 cells was inhibited with actinomycin D and the stability or lifespan of the message was determined over 6 hours. The conditions for these experiments were identical to those described in chapter 5 and the mRNA levels were measured both in the presence and the absence of serum due to the reasons given in chapter 5.

The results of the normal time course in the presence of serum (figure 7.2) show that PAI-1 is expressed in A15A5 with a rapid rise in message levels over the initial 4 hours. This rapid induction within the initial 4 hours in the presence of serum was also seen in other time courses in this study (figure 7.5,7.8).

The time course with actinomycin D shows that inhibition of transcription leads to a sudden elevation in the PAI-1 message level though the transcripts then undergo gradual degradation subsequently. The level of the message however remain relatively high over 6 hours.



0 2 4 6 2+ 4+ 6+

PAI-1

+ = actinomycin D

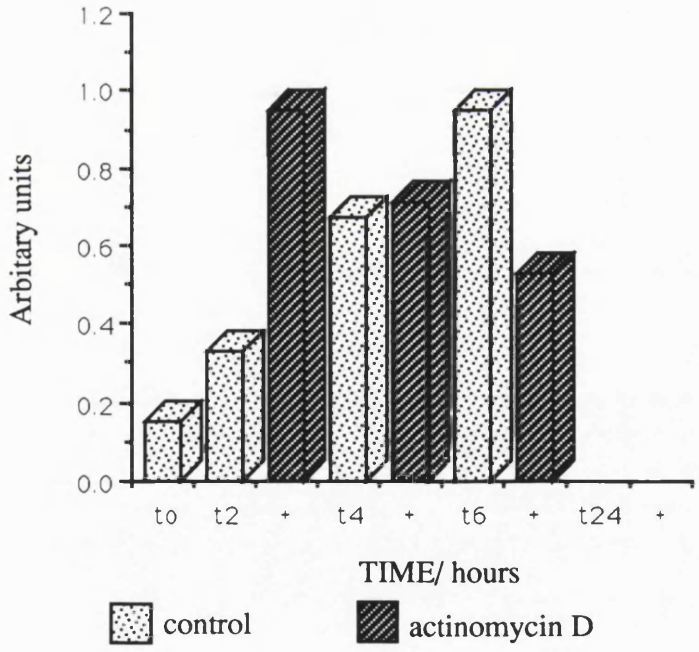
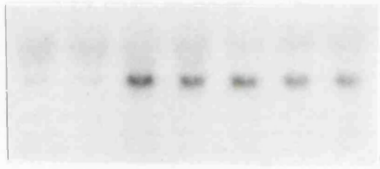


Figure 7.2 : A time course of PAI-1 mRNA after inhibition of transcription in the presence of serum.



6 4 2 0 2+ 4+ 6+

tPA PAI-1

+ = actinomycin D

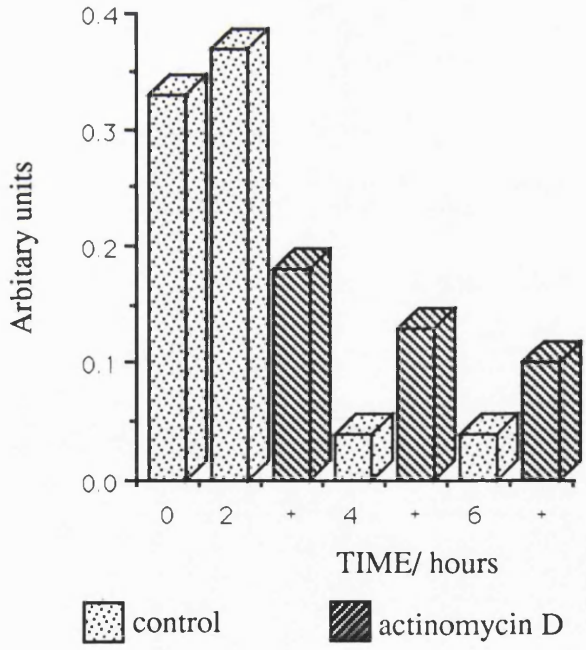


Figure 7.3 : A time course of PAI-1 mRNA after inhibition of transcription in the absence of serum.

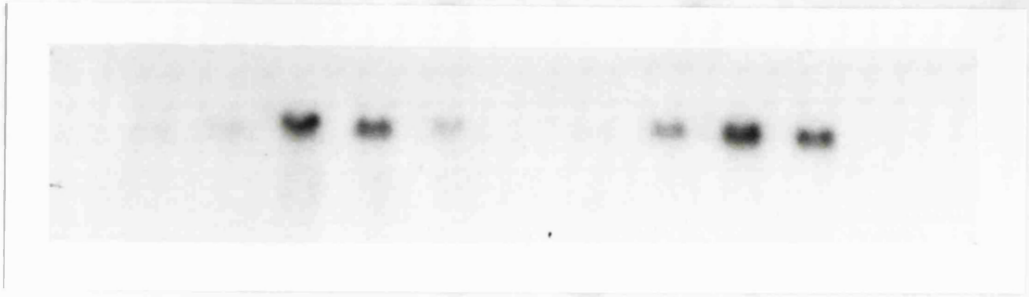
The figure 7.3 show the time course of PAI-1 in the absence of serum. As discussed earlier expression in the absence of serum relates to the conditions under which CM is collected for fibrinolysis assays. Therefore stability of the mRNA in the absence of serum was measured to determine whether PAI-1 levels can be suppressed in A15A5 in the absence of serum. Work by other groups have established that the PAI-1 activity can be suppressed in the absence of serum partly due to conversion of active protein to its latent form (Heckman and Loskutoff, 1985).

Results (figure 7.3) suggest that in contrast to its stability in the presence of serum the PAI-1 message is unstable in the absence of serum. The normal time course indicate that the mRNA undergo rapid decline by 4 hours. However after inhibition of transcription by actinomycin D the message is stabilised or degraded at a slower rate compared to the normal levels.

7.5.2b The regulation of PAI-1 mRNA after inhibition of translation.

Earlier it was discovered that inhibition of translation leads to the increased degradation of the tPA mRNA after 6 hours. This result contradicts the proposed theory that mRNA with AUUUA pentamers within their 3'UTR is stabilized upon translation inhibition due to suppression of proteins that target AU - rich sequences for rapid degradation. PAI-1 mRNA also has two AUUUAs in its 3'UTR, and indeed many workers have demonstrated that cycloheximide could stabilise PAI-1 mRNA (Lucore et al., 1988, Georg et al., 1989). However some studies have indicated that translation inhibition could also suppress PAI-1 levels, suggesting that the effect of translation inhibition on PAI-1 mRNA may be cell or tissue specific.

Therefore a time course study was carried out to determine the effect of cycloheximide on the expression of PAI-1 message in the A15A5 cell line. The cells were treated with cycloheximide under the same conditions described in chapter 5. Figure 7.4 shows the effect of translation inhibition in the presence of serum on PAI-1 mRNA expression. The normal time course again showed an elevation of the message by 4 hours which subsequently undergo degradation. However, cycloheximide was found to augment this rise in levels. A duplicate experiment confirmed this significant rise in the PAI-1 message level by 4 hours (not shown). In both these experiments there was a 3-4 fold elevation in the mRNA level at 4 hours after addition of cycloheximide. However the message is not stable, it undergoes



0 2 4 6 8 2+ 4+ 6+ 8+

Time (hours)

PAI-1

+ = cycloheximide

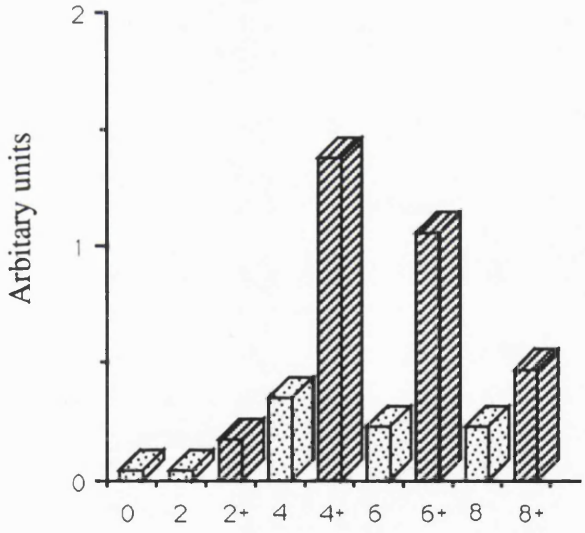


Figure 7.4 : The stability of PAI-1 mRNA in A15A5 after inhibition of translation in the presence of serum.

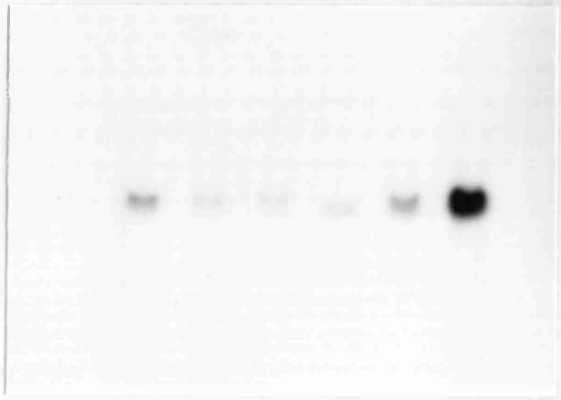
Control Cycloheximide (10µg/ml)

degradation afterwards. In the repeat the message levels fell to basal levels by 8 hours. These results suggest that either there is a transient elevation of transcription or a transient suppression in the degradation of the PAI-1 mRNA during translation inhibition.

The rapid rise in mRNA level also resemble kinetics of superinduction seen with immediate-early genes. It has been demonstrated by several groups including Edwards and Mahadevan (1992) that the addition of protein synthesis inhibitors simultaneously with stimulation of growth leads to a rapid and prolonged transcription (superinduction) of c-fos and c-myc protooncogenes.

Therefore a time course of PAI-1 in ARBO C9 was investigated to determine the expression of its mRNA in the presence of serum and whether the kinetics of expression after translation inhibition are similar to that in A15A5.

The time course in figure 7.5 show the level of PAI-1 mRNA in ARBO C9 over 6 hrs under normal growth conditions and after translation inhibition. The normal time course show that the message remain stable and even rises moderately during this time period. However, translation inhibition leads to a further increase in the level of the message, as it remain high over 6 hours. Therefore these results contrast to the expression of PAI-1 in A15A5 where the mRNA rises and then undergo degradation. The results suggest that PAI-1 is much more stable in ARBO C9 than in A15A5 in the presence of cycloheximide.



0 2 2+ 4 4+ 6 6+

Time (hours)

PAI-1

+ = Cycloheximide

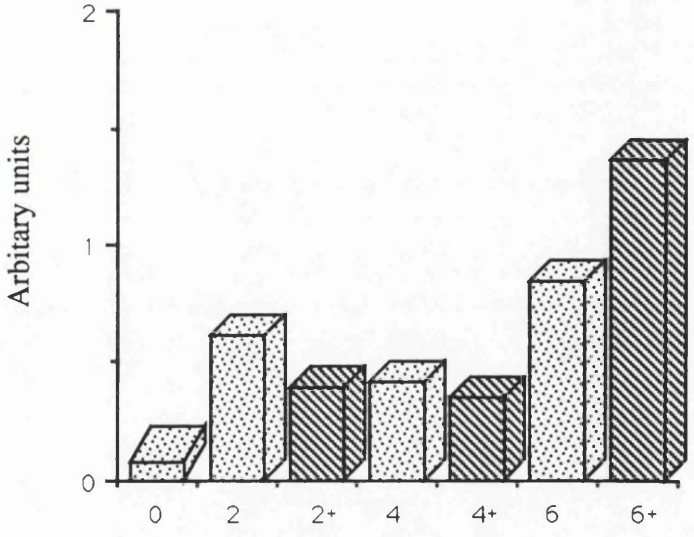


Figure 7.5 : Expression of PAI-1 mRNA in ARBO C9 after inhibition of translation in the presence of serum.

Control Cycloheximide (10µg/ml)

7.5.3 The nuclear and cytoplasmic distribution of PAI-1 mRNA.

The results from the nuclear run-on assay showed that PAI-1 is transcribed at an approximately identical rate in both cell lines. These results are comparable to the results obtained for tPA where the level of transcription between the cell lines was almost identical. Despite this similarity in transcription, the elevated tPA mRNA level in the glioma cells was found to be primarily due to increased stability of the transcript in the nucleus of A15A5. Therefore the stability of PAI-1 mRNA in the nucleus and the cytoplasm was measured to ascertain whether there is a difference in the stability of PAI-1 in A15A5 and ARBO C9 and to determine whether the difference is seen at the nuclear or cytoplasmic level of the cell.

The RNA levels were measured in cells growing in 15% serum. The figures 7.6a and 7.6b show the nuclear and cytoplasmic distribution of PAI-1 in A15A5 and ARBO C9. There are high levels of PAI-1 in both the nuclear and the cytoplasmic fractions of ARBO C9, while in A15A5 the PAI-1 RNA is considerably low in both fractions. In ARBO C9 the cytoplasmic message level was greater than the nuclear level which is a marked contrast to tPA mRNA in these cells.

Later the nuclear mRNA level was measured 4.5 hours after change of medium. This time point was chosen because the time course experiments indicated (figures 7.2, 7.4) that maximal level of PAI-1 mRNA was expressed between 2.5 to 8 hours after change of medium. Figure 7.6b shows that PAI-1 is

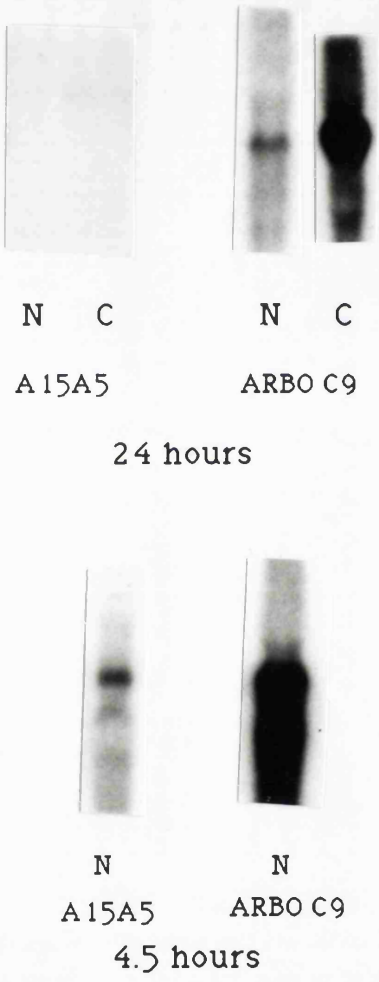


Figure 7.6a: Nuclear and cytoplasmic distribution of PAI-1 RNA in A15A5 and ARBO C9

N = nuclear ; C= cytoplasmic

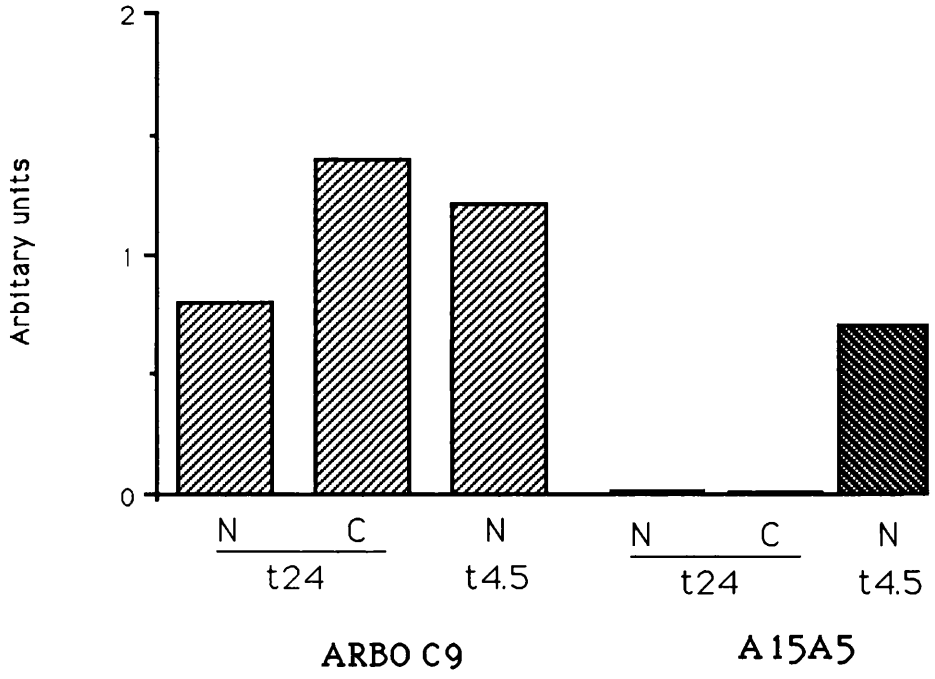


Figure 7.6b Graph of standardised nuclear and cytoplasmic PAI-1 mRNA levels .
t= time

elevated at 4.5 hours in the nucleus of both A15A5 and ARBO C9. However the rise was greater in ARBO C9 than in A15A5. Unfortunately cytoplasmic levels of the PAI-1 could not be determined due to the degradation of cytoplasmic RNA preparations from these cells.

These results suggest that the constitutive PAI-1 mRNA level is higher in ARBO C9 despite the similarity in the level of transcription of PAI-1 in A15A5 and ARBO C9. Furthermore they also show that addition of fresh medium leads to an induction of PAI-1 in the glioma cell line. Therefore differential expression of PAI-1 may account for some of the effects that extrinsic agents that induce cellular signalling have on PA activity of the glioma cells.

Several workers have found that PAI-1 could be expressed simultaneously with tPA in the same cells, including tumour cells. Whilst some studies have found that PAI-1 is suppressed in transformed cells whilst tPA is elevated. These data suggest that PAI-1 message could be differentially regulated in a cell specific manner.

Therefore the regulation of PAI-1 in A15A5 was further investigated to compare its regulation to that of tPA and furthermore, to gain an insight into the differential regulation of two transcripts that have similar transcription rates but different mRNA levels in the same cell line.

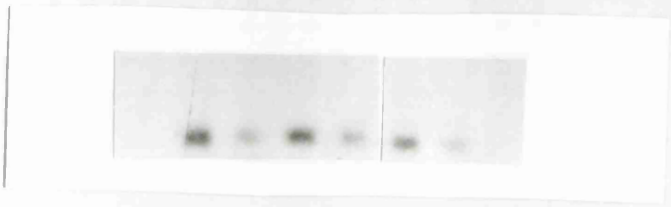
7.5.4. MODULATION OF PAI-1 mRNA BY INDUCERS OF INTRACELLULAR SIGNALLING.

7.5.4a. The effect of Cholera toxin on the expression of PAI-1 mRNA in A15A5.

Cholera toxin was shown to elevate tPA mRNA levels in A15A5. Induction of intracellular cAMP is known to induce tPA mRNA in several cell lines, while other studies have demonstrated that induction of intracellular cAMP leads to a suppression of PAI-1 activity. Ny et al. (1985) showed leutinising hormone (LH) and follicle stimulation hormone (FSH), both of which induce cAMP suppress PAI-1 activity in FSH primed rat granulosa cells, while Andreasen et al. (1986a) showed dibutryl-cAMP, an analogue of cAMP also inhibit PAI-1 activity.

Other workers have however demonstrated that induction of cAMP can also cause small increases in PAI-1 antigen and mRNA levels. Santell and Levin (1988) found cAMP causes a 2-fold rise in PAI-1 antigen levels in CM of human umbilical vein endothelial cells. Studies by Heaton et al. (1989) showed CPT-cAMP (8-4-chlorophenylthio cAMP) also an analogue of cAMP to elevate PAI-1 mRNA by 2-fold in primary rat hepatocytes.

Therefore cholera toxin modulated expression of PAI-1 mRNA in the A15A5 cell line was investigated in both the presence and absence of serum. The figure 7.7 shows the level of PAI-1 mRNA 24 hours after addition of cholera toxin to the culture



PAI-1

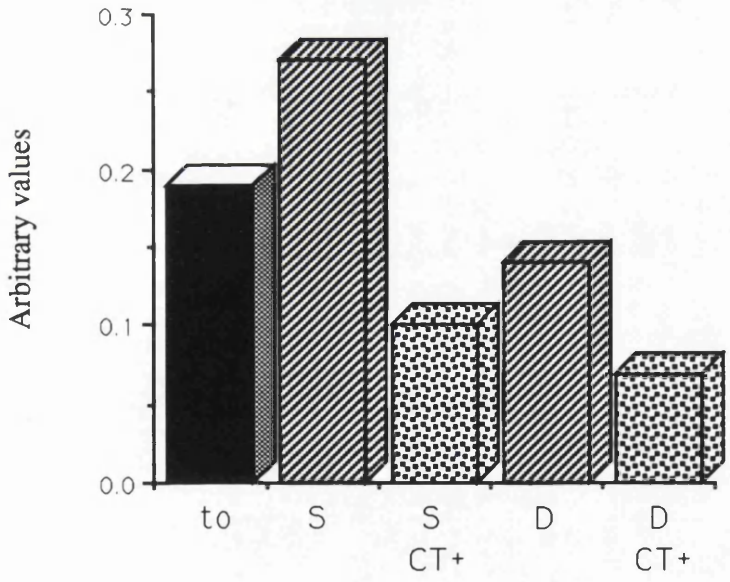


Figure 7.7 The effect of cholera toxin on the expression of PAI-1 mRNA after 24 hours

S= 15% Serum ; D= 0% serum ; CT= Cholera toxin ; 24 hours unchanged medium not shown.

medium. As can be seen from the results cholera toxin causes an inhibition of the level of PAI-1 message in both the presence and the absence of serum.

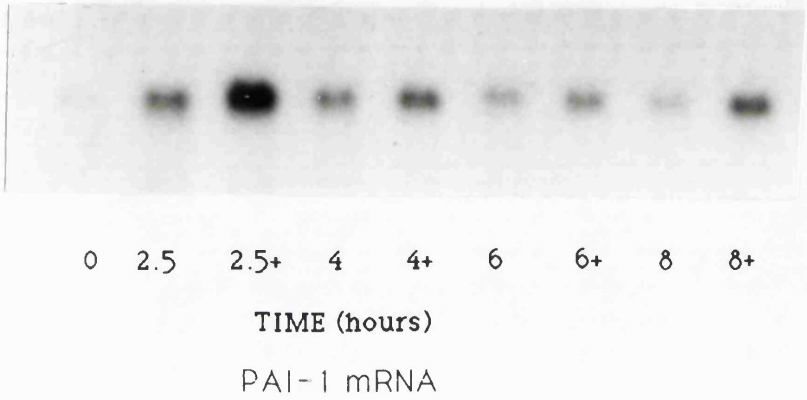
The figures for the normal levels also confirm the earlier findings that serum can induce PAI-1 mRNA in A15A5, whilst in the absence of serum the message is considerably decreased.

7.5.4b. The effect of dexamethasone on the expression of PAI-1 mRNA in A15A5.

The glucocorticoid dexamethasone is a known suppressor PA activity in many cell lines. Carlson & Gelehrter showed in 1977 that glucocorticoids could suppress plasminogen dependent fibrinolytic activity in the HTC rat hepatoma cell line.

Later it was demonstrated that dexamethasone could induce both PAI-1 protein and mRNA levels. Dexamethasone mediated elevation of PAI-1 protein levels were demonstrated for several cell lines including human embryonal lung cell line Hel 299 (Lund et al., 1988), fibrosarcoma cell line HT 1080 and the human glioblastoma cell line UCT/gl1 (Andreasen 1986a,1987). The elevation of PAI-1 levels by dexamethasone was not universal, as in several other cell lines dexamethasone was found to have no effect on PAI-1 (Lund et al., 1988).

Previous studies demonstrated that 10^{-7} dexamethasone could almost abolish the constitutively expressed high PA activity in



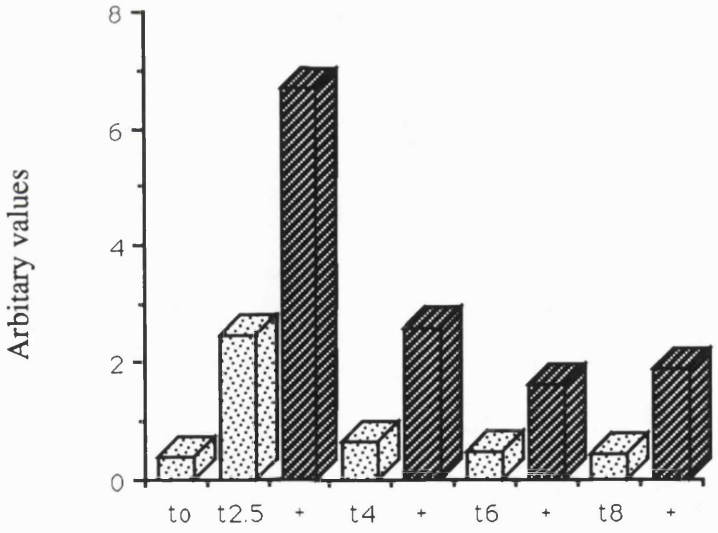


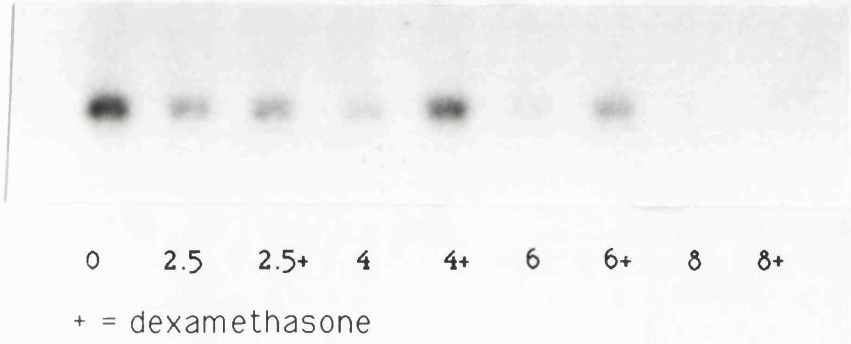
Figure 7.8 : Induction of PAI-1 by dexamethasone in A15A5 in the presence of serum

+ = Dexamethasone

A15A5 cells. However in this study (chapter 6) it was found that dexamethasone does not profoundly suppress the expression of tPA mRNA in A15A5.

As other workers have demonstrated that dexamethasone could induce PAI-1 mRNA and its activity, a time course of PAI-1 mRNA was carried out to determine whether the dexamethasone mediated suppression of PA activity could be due to the induction of PAI-1 in A15A5. The expression of PAI-1 was examined in both the presence and the absence of serum. The expression mRNA in the presence of serum was investigated to determine if dexamethasone can induce PAI-1 mRNA in A15A5 cells. As it was found that PAI-1 mRNA in A15A5 cells are degraded rapidly when serum is absent from the medium, the effect of dexamethasone on the PAI-1 message in the absence of serum was analysed to determine whether PAI-1 mRNA can be induced by dexamethasone even in the absence of serum.

The figure 7.8 shows the the effect of dexamethasone on the expression of PAI-1 mRNA in the presence of serum. The results indicate that there is a rapid and a considerable induction in the level of PAI-1 mRNA by dexamethasone. After this rapid induction the message begins to decline, but the levels still remain greater than the basal level over 8 hours. These results indicate that dexamethasone can induce PAI-1 mRNA in the A15A5 cell line.



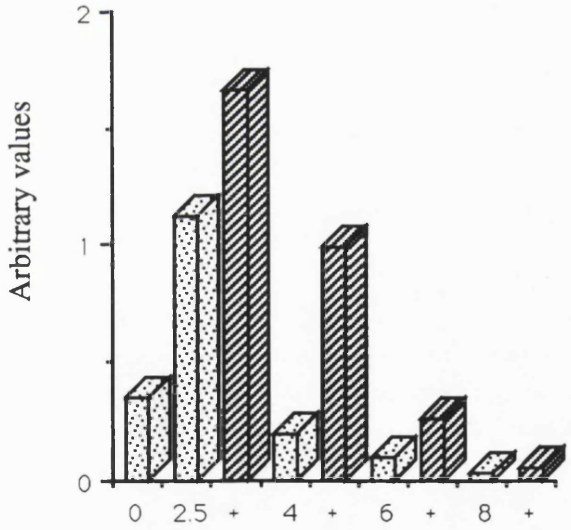
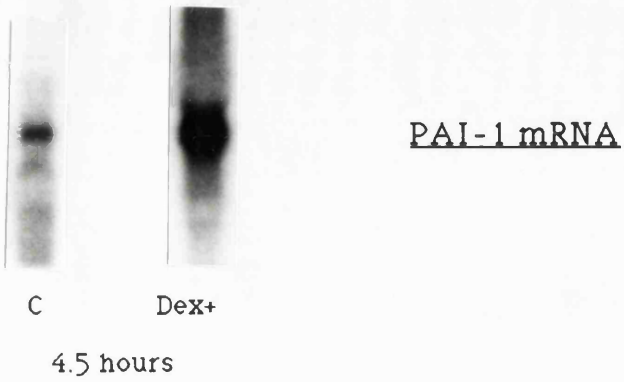


Figure 7.9 : Induction of PAI-1 by dexamethsone in A15A5 in the absence of serum.

The expression of the PAI-1 message in the absence of serum is shown in figure 7.9. The time course of the normal message showed the kinetics of mRNA degradation in the absence of serum observed earlier (figure 7.3). In the presence of dexamethasone the PAI-1 message was considerably elevated upto 4 hours. However, the message is again found to be unstable in A15A5 and decline to basal levels by 8 hours.

The level of the PAI-1 message in the nuclei of A15A5 cells 4.5 hours after administration of dexamethasone was also determined. Earlier it was found that PAI-1 mRNA could be hardly observed in the nuclei of log phase A15A5 cells. However it was decided to determine if dexamethasone mediated elevation in the total PAI-1 message could also be seen in the nuclei of A15A5.

Figure 7.10 shows the nuclear PAI-1 mRNA levels at 4.5 hours before and after induction with dexamethasone in the presence of serum. The results show an increased nuclear PAI-1 mRNA levels in the presence of dexamethasone. However the difference in the levels are not as great as seen with total mRNA level where the difference was approximately 3 fold at 4 hours.



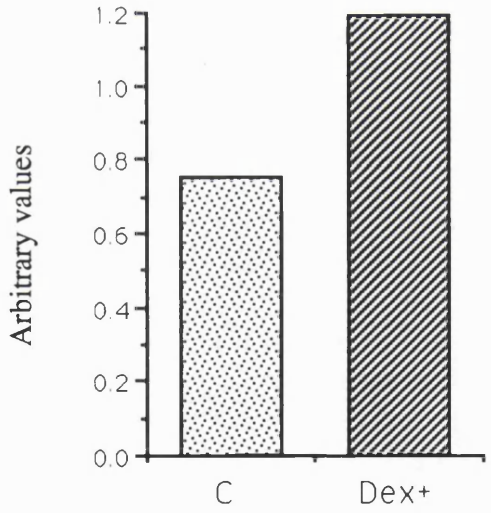


Fig 7.10 Nuclear PAI-1 mRNA levels at 4.5 hours in A15A5 cells after induction by DEX.

C= control ; Dex+ = dexamethasone.

7.6. DISCUSSION

The expression of PAI-1 in A15A5 and ARBO C9 cells was studied to obtain a possible explanation for the large difference in PA activity between the cell lines. Studies on tPA mRNA levels showed that disparity in tPA message level by itself cannot explain the 50-80 fold difference in PA activity between the cell lines. PAI-1 is the principal inhibitor of tPA activity in tissue. It reacts rapidly with tPA to inhibit its activity. Therefore it was hypothesized that the PAI-1 mRNA may be expressed constitutively at a higher level in ARBO C9 or that the PAI-1 message is suppressed in A15A5.

Nuclear run-ons were carried out to determine the rate of transcription of PAI-1 in A15A5 and ARBO C9. The run-on assays indicated that PAI-1 is transcribed at a similar rate in both cell lines and its rate of transcription is lower than that of tPA in these cells.

The similarity in the level of transcription of PAI-1 is comparable to the kinetics of transcription of tPA in A15A5 and ARBO C9. However post-transcriptionally elevated nuclear stability of the message was found to be the primary course of the higher level of tPA mRNA in the glioma cell line.

In contrast to tPA, PAI-1 mRNA was found to be constitutively expressed in the normal cell line, while it was difficult to detect in both the cytoplasm and the nucleus of unstimulated A15A5

cells. The results show major differences in the mechanisms of regulation of constitutive levels of these transcripts between A15A5 and ARBO C9.

Further analysis of the expression of PAI-1 mRNA in A15A5 found that PAI-1 could be expressed in A15A5, but the message is less stable than tPA in this cell line.

A time course of PAI-1 mRNA after transcription inhibition by actinomycin D in the presence of serum indicated that the PAI-1 transcript is more stable when serum is present in the medium. The results also found that inhibition of transcription leads to a rapid transient elevation of the message. There are two possible causes for this effect. There may be a labile repressor of PAI-1 transcription which is removed upon transcription inhibition or that there is a reduction in the rate of degradation of the message.

In the absence of serum the level of PAI-1 was found to fall substantially after 4 hours. The reason may be either depression of its transcription or increased destruction of its mRNA. Transcription inhibition was found to reduce the rate of decline or make the message more stable, which indicate that lowered level of PAI-1 in the absence of serum is due to increased degradation of the message. These results also indicate that the PAI-1 message is generally unstable in A15A5 cells. The difference in the level of decline in the presence and in the absence of serum suggest that serum either elevates

transcription of PAI-1 in A15A5 or that in its presence a specific factor is expressed that protects PAI-1 message from degradation.

These results are in contrast to the expression of tPA in A15A5 where it was found that tPA is stable in these cells with little degradation taking place over a 6 hour period.

Several other studies on PA activity of both normal and tumour cells have also demonstrated this paradoxical nature of expression of PA and their inhibitors. Quax et al. (1990) found that both PAs and their inhibitors could be expressed simultaneously by tumour cells. Furthermore they found that those tumours that expressed highest levels of PAs also to be expressed highest levels of the inhibitors.

In A15A5 glioma cells, translation inhibition by cycloheximide leads to a dramatic increase in PAI-1 mRNA levels at 4 after treatment. The levels then gradually decline over next 4 hours and reaches basal levels by 24 hours. This considerable rise in PAI-1 message was not seen in nuclear RNA levels, where the PAI-1 message in cycloheximide treated nuclei was similar to untreated levels, which suggest the effect of cycloheximide is due to increased stability of cytoplasmic RNA.

The effect of cycloheximide on PAI-1 is opposite to its effect on tPA where cycloheximide treatment suppresses tPA message levels. Cycloheximide was also found to cause no difference in

the nuclear tPA RNA levels, again showing that its effect must be at the cytoplasm possibly through stability of the message.

The mechanism involved in the increased stability of message after translation inhibition has been proposed to be due to suppression of trans-acting factors that recognise specific sequences such as the ARE in the 3' UTR and target their destruction. Several authors have demonstrated increase in message stability of 3' ARE containing transcripts upon translation inhibition.

Inducers of cAMP have been shown to be inhibitors of PAI-1 activity in some cell lines. LH and FSH both of which induce cAMP levels suppress PAI-1 activity of FSH primed rat granulosa cells (Ny et al., 1985). Inducers of intracellular cAMP have also been found to suppress weakly PAI-1 activity of CM from UCT/gl-1 glioma cells (Andreasen et al., 1986b).

In this study cholera toxin was found to inhibit PAI-1 mRNA levels in 15% serum and in the absence of serum 24 hours after treatment. These results further confirm the earlier results that removal of serum from the growth medium cause a rapid decline in the PAI-1 message levels. The level of inhibition of PAI-1 by cholera toxin was similar under both conditions. Which suggest that cholera toxin may suppress basal level of expression of PAI-1. Cholera toxin is known to modulate genes through induction of mRNA transcription

through activating the transcription factor (CREB) that binds to the cAMP response element (CRE) of promoters.

How cholera toxin can directly inhibit transcription of genes like PAI-1 that lack a CRE its promoter is unknown.

Glucocorticoids are well known inducers of PAI-1 protein and mRNA levels and thus inhibitors of PA activity of several cells. Dexamethasone was found to induce PAI-1 mRNA in both the absence and presence of serum in a similar manner. Under both conditions there is an early and rapid rise in PAI-1 mRNA in the A15A5 cells, which subsequently declines. However, in the absence of serum the rate of degradation was much quicker, where the induced PAI-1 mRNA levels decline to basal levels by 8 hours. While in the presence of serum the PAI-1 message levels remain higher than the basal level upto 8 hours and possibly beyond.

The effect of dexamethasone was seen in both the total and nuclear RNA levels which were both elevated above normal.

These studies on the expression of PAI-1 mRNA showed that some of the dexamethasone mediated abolition of PA activity in glioma cells reported in previous studies are probably due to its effects on PAI-1 levels. In this study it was found that dexamethasone does not suppress tPA mRNA both in the presence and absence of serum. However it strongly induces PAI-1 mRNA levels under both conditions.

These results agree with some of the other published data where dexamethasone has been shown to elevate PAI-1 mRNA levels in several cell lines.

There is a stark contrast in the expression of PAI-1 and tPA in the two cell lines. In A15A5 cells PAI-1 transcripts were difficult to detect both in the nucleus and the cytoplasm of "cycling" cells. Whilst high levels of PAI-1 mRNA were found in both the nucleus and cytoplasm of ARBO C9 cells, which suggests that PAI-1 is constitutively expressed at high levels in the normal cell line.

However PAI-1 could be detected in the A15A5 nuclei at 4.5 hours after medium change which can be further induced by treatment with dexamethasone. Unfortunately cytoplasmic mRNA levels in these cells at 4.5 hours could not be detected due to the inability to obtain clean undegraded cytoplasmic mRNA from the cells.

CHAPTER 8 : CONCLUDING DISCUSSION.

Elevated tPA mediated fibrinolytic activity has been shown to be a characteristic of several virally and chemically transformed cell lines and in cells derived from naturally occurring tumours. Regulated expression of increased tPA is also found in several normal physiological events such as oogenesis and wound healing. It has been proposed that constitutively elevated PA levels in neoplastic cells may have a role in extracellular tissue degradation during invasion and metastasis of tumours.

Previous studies have established that the A15A5 cell line expresses higher levels of both tPA mediated fibrinolytic activity and tPA mRNA compared to its normal counterpart ARBO C9. The A15A5 cell line was established from an ENU induced brain glioma, whilst ARBO C9 is a cell line derived from a normal rat brain.

The fibrinolytic activity and tPA mRNA levels are 50 - 80 fold and 10-20 fold higher respectively in A15A5 compared to ARBO C9. The PA activity of the A15A5 cell line was also found to be influenced by several extrinsic agents that modulate intracellular signal transduction. Two of these agents, cholera toxin and EGF was found to elevate PA activity of conditioned medium from A15A5 cells. Cholera toxin was also found to enhance PA activity of A15A5 cell lysates.

Another agent dexamethasone was shown to suppress PA activity of both conditioned medium and lysate from A15A5.

This project was primarily concerned with analysing two aspects of the expression of tPA in the two cell lines at the molecular level:

i. To investigate the regulation of tPA mRNA in the cell lines and to determine the cause of elevated tPA mRNA levels in the glioma cell line.

ii. To investigate the effect of extrinsic agents on the mRNA levels of tPA and its inhibitor PAI-1.

A 1700 base pair fragment of tPA cDNA which included the whole of the 3'UTR was sequenced from each of A15A5 and ARBO C9 cell lines. Mutations in both the coding region and the 3'UTR have been shown to influence stability and degradation of other messages. However no differences were found in the tPA cDNA sequences from the two cell lines.

Nuclear run-on assays were carried out to determine the level of transcription of tPA between A15A5 and ARBO C9. The run-on assays showed that tPA is transcribed at a comparable rate in both the cell lines. Several repeats of the assays indicated that the level of transcription was only 1.5 - 2 fold higher in A15A5.

Thus the rate of transcription of the message did not account for the difference in the mRNA level between the cell lines.

The markedly lower level of the message found in previous experiments with ARBO C9 indicated that tPA mRNA may be unstable in the normal cell line, furthermore it also precluded a direct comparison of stability between the cell lines. Therefore stability of the transcript in A15A5 was investigated to determine whether an increase in its stability could explain the higher level of the message in A15A5. Experiments were performed using cells maintained in usual growth medium with serum and in medium without serum to mimic conditions under which CM is collected to measure PA activity.

A time course of the expression of tPA after transcription inhibition indicated that tPA mRNA is stable up to 6 hours in the presence of serum. The message was also detected in the absence of serum, but there is a gradual decline over this period suggesting that tPA transcripts are less stable in the absence of serum.

The 3'UTR sequences of tPA from A15A5 and ARBO C9 matched completely the published rat tPA sequence. There was no difference found in the 3'UTR of tPA of the two cell lines. However there are two AUUUA pentamers within a region high in AU nucleotides (ARE) in the 3' UTR of tPA. AUUUAs and AREs have been demonstrated in several studies (Kabnick and Houseman, 1988; Malter, 1989) to regulate mRNA stability. It has been proposed that these elements target mRNA for rapid degradation through specific ARE binding

trans-acting proteins. For a number of transcripts containing ARE sequences inhibition of translation was found to lead to an elevation of their message levels. Therefore the expression of tPA in A15A5 was determined in the presence of a translation inhibitor (cycloheximide). It was found that inhibition of translation does not lead to an elevation of the tPA mRNA levels in A15A5, however the message remain stable upto 6 hours.

PAI-1 is the major tissue specific inhibitor of tPA and uPA proteins. Thus it has a principal role in the regulation of PA mediated plasminogen activation.

Exponentially growing A15A5 cells were found to express substantially lower level of PAI-1 mRNA compared to ARBO C9. Nuclear run-on assays showed that PAI-1 mRNA is transcribed at an almost identical rate in A15A5 and ARBO C9, indicating that the difference in message level between the cell lines is due to a post -transcriptional event.

Inhibition of transcription in the presence of serum was found to cause a transient induction of PAI-1 mRNA in A15A5. Subsequently the transcripts undergo gradual decline over the next 6 hours suggesting that PAI-1 is somewhat less stable than tPA in A15A5.

Meanwhile in the absence of serum PAI-1 was found to undergo rapid degradation. Inhibition of transcription leads to

the slowing of the rate of decline or an increase in the the stability of the message.

These data suggest that PAI-1 may be naturally unstable and targeted for destruction in A15A5 cells. The increased PAI-1 mRNA in the presence of serum is partly responsible for the suppression of total PA activity demonstrated in several cell lines. The increased PAI-1 levels in the presence of serum may be partly due to the induction of PAI-1 mRNA by TGF ß, one of the growth factors present in serum. TGF ß has been demonstrated to induce PAI-1 mRNA in several cell lines including human lung fibroblast cell line (Laiho et al., 1986). The authors also found EGF could overcome TGF ß mediated suppression of PA activity in conditioned medium from these cells due to the even greater elevation of PAs.

The published rat PAI-1 mRNA sequence show two AUUUA pentamers, also within a region rich in AU nucleotides. Translation inhibition in the presence of serum leads to a considerable elevation of PAI-1 mRNA in A15A5. These results agree with the proposed ideas for the effect of translation inhibition on mRNAs containing AU-rich elements in their 3'UTR. However they contrast with the data obtained for stability of tPA mRNA which also has two AUUUA pentamers.

The context of the two AUUUA pentamers within the ARE of PAI-1 is different to the published tPA ARE. Although the

PAI-1 mRNA sequence from A15A5 and ARBO C9 was not determined, the results suggest that the two cell lines could distinguish between the AREs or sequences that are involved in message degradation and regulate the two transcripts differentially despite the comparable levels of transcription of the mRNA. This would be similar to what takes place during the regulation of tPA mRNA between A15A5 and ARBO C9. However, it is also possible that there is an alteration in the PAI-1 sequence between the cell lines and that is the cause of the difference in its expression between A15A5 and ARBO C9.

The nuclear and cytoplasmic distribution of tPA and PAI-1 RNA in A15A5 and ARBO C9 was also investigated. It was discovered that the tPA RNA level was considerably higher in the nucleus than in the cytoplasm of A15A5 cells, whilst it was barely detectable in both nuclear and cytoplasmic fractions of ARBO C9. This was an important observation as it suggested that the elevated level of the tPA mRNA seen in the A15A5 cell line is primarily due to the increased nuclear stability of the message.

In contrast to tPA, RNA for PAI-1 was found to be present at high levels in both the nucleus and the cytoplasm of ARBO C9 cells. In A15A5, although the constitutive level of PAI-1 was low in both the nucleus and the cytoplasm, it is transiently induced when fresh growth medium is added to the cultures.

The cause of the elevated nuclear stability of tPA mRNA in A15A5 appear to be due to an epigenetic event. It may involve trans-acting factors such as ARE- binding proteins that regulate message stability. Several AUBPs have been identified in other cells that are present in both the nucleus and the cytoplasm. There may be differences in the expression of AUBPs in A15A5 and ARBO C9 that are responsible for this difference. Therefore, it can be proposed that during neoplastic transformation of the glioma cells there has been a possible alteration in the expression of AUBPs in the cells.

The finding that increased stability of the nuclear transcript is responsible for the post-transcriptional regulation of tPA in A15A5 is not unprecedented, however it is an important discovery as there are only a few reported examples of RNA stability being regulated in the nuclei of cells. Other reports of nuclear regulation include the mRNA for uPA (Henderson and Kefford, 1991), dihydrofolate reductase (DHFR) (Leys et al., 1984), α1-acid glycoprotein (AGP) (Vannice et al., 1984) and both α and ß subunits of T-cell receptor (TCR) (Wilkinson and MacLeod, 1988).

The uPA mRNA level is 26-fold higher in the DMBA induced metastatic rat mammary adenocarcinoma cell line MAT13762 compared to that in the rat embryo fibroblast cell line rat-1. These differences are found despite the transcription levels of the message being identical between the cell lines. The reason for the difference in the uPA level was identified as due to

post-transcriptional downregulation of the message in the nucleus of the rat-1 cells or due to the increased stability of nuclear transcript in the MAT13762 cells (Henderson and Kefford, 1991).

The level of the uPA mRNA in MAT13762 cells was also high compared to that in the spontaneously induced and highly metastatic rat mammary adenocarcinoma cell line BC1. The nuclear transcript level was 14-fold higher in the MAT13762 cells than in the BC1 nuclei (Henderson et al., 1992). The invasive property of the BC1 cells was related to the overexpression of the transin gene, a matrix metalloprotease rather than to uPA activity in the cells.

Furthermore, translation inhibition with cycloheximide elevated the steady state level of the nuclear transcript of uPA in BC1 cells. A similar affect on the nuclear levels of α and ß subunits of T-cell receptor has also been reported (Wilkinson and MacLeod, 1988). Nuclear precursor-RNA for both of the subunits could be detected in the nuclei of murine T-lymphoma cell line SL12.4, although an appreciable quantity of the mature TCR mRNA does not accumulate in these cells. However treatment of the cells with cycloheximide leads to the elevation of both α and ß messages in the nucleus and the cytoplasm of the SL12.4 cells. The cytoplasmic mRNA probably represents the exported mature mRNA, but the underlying reason for this is likely to be decreased degradation or the greater maturation of the nuclear transcript.

Protein synthesis was also required for the dexamethasone mediated induction of α1-acid glycoprotein transcripts in the nuclei of HTC rat hepatoma cells (Vannice et al., 1984). In the absence of dexamethasone, mature α1-acid glycoprotein mRNA cannot be seen in these cells despite the detectable transcription rate of the gene. When the cells are treated with dexamethasone the nuclear level of the message increases substantially.

These studies indicate that cells can actively downregulate maturation of hnRNA in the nucleus. Addition of the translation inhibitor cycloheximide was reported to lead to the elevation of uPA, TCR and AGP mRNA in the nuclei of corresponding cell lines suggesting that there is a constitutively expressed labile protein that target their pre-RNA to be degraded in the cells. Whether a similar mechanism is involved in the low basal level of the tPA mRNA in ARBO C9 cells and an alteration in this normal mechanism is responsible for the elevated level of the message in the glioma cells is yet to be investigated.

Three extrinsic agents cholera toxin, EGF and dexamethasone have been previously demonstrated to influence PA activity of A15A5 cells. These agents have also been demonstrated to modulate mRNA levels of PAI-1 in other cell lines. Cholera toxin was found to induce tPA in both the presence and in the

absence of serum, while it downregulated PAI-1 mRNA levels under both conditions. Previously it was shown that cholera toxin elevates tPA mediated PA activity by 2-fold in conditioned medium from A15A5. This is primarily due to its influence on the tPA mRNA level, which was elevated by approximately 1.5-fold by cholera toxin in the absence of serum.

Dexamethasone was shown previously to suppress PA activity in A15A5 by as much as 5-fold. Dexamethasone was found to have no profound effect on the tPA mRNA levels whether in the presence or in the absence of serum. However it was found in this study that dexamethasone causes a substantial increase in PAI-1 mRNA levels under both conditions. Therefore it can be concluded that dexamethasone mediated considerable depression of PA activity seen in the A15A5 cell line is principally due to its effect on PAI-1 message.

Similar results have also been reported by several other workers. Dexamethasone was found to suppress fibrinolytic activity of conditioned medium from HT1080 human fibrosarcoma cells to below detectable levels by 24 hours (Medcalf et al., 1988). There was a 10-fold increase in PAI-1 antigen level with a simultaneous 2-fold rise in tPA levels. Analysis of mRNA levels indicated a 3.4-fold increase in tPA mRNA, but also a 11.9- and 7.8-fold increases in 3.4 kb and 2.4 kb forms of human PAI-1 mRNA respectively. Dexamethasone also caused a greater than 95% decrease in uPA mRNA levels

by 8 hours. Run-on assays indicated that dexamethasone induces PAI-1 mRNA transcription over 24 hours. Transcription of tPA was induced, but only by a maximum of 3- to 4- fold between 4 and 8 hours, which falls by 24 hours.

Furthermore, treatment with cycloheximide resulted in a substantial increase in PAI-1 level between 4 and 8 hours while only causing a modest increase in tPA mRNA level during this period.

Dexamethasone was also found to substantially suppress (by 90%) PA activity in HTC rat hepatoma cells (Heaton and Gelehrter, 1989), while 8-Bromo cAMP an analogue of cAMP was shown to elevate PA activity by 50% in the same cells (Barouski-Miller et al., 1990). Later Heaton et al., (1992) demonstrated using nuclear run-on assays that dexamethasone causes a significant increase (5-fold) in transcription of PAI-1 mRNA, while causing only a modest increase of 2-fold in the transcription of tPA. Furthermore 8-Bromo cAMP was shown to inhibit transcription of PAI-1 by 60% while inducing the transcription of tPA by approximately 2-fold.

Studies by Heaton et al (1992) on mRNA stability reported that transcription inhibition has no effect on the decay of the PAI-1 message. Dexamethasone was also found to cause no change in decay of PAI-1 mRNA. However cAMP was found to depress the half-life of PAI-1 by 3 fold.

The results from these studies on HT1080 and HTC cell lines resemble several aspects of the regulation of tPA and PAI-1 seen in A15A5 cells. Principally, the inhibition of high fibrinolytic activity by dexamethasone in both human fibrosarcoma cell line HT1080 and the rat HTC hepatoma cell line is due to elevation of the PAI-1 message and not due to a decline in the tPA mRNA levels. Furthermore in the rat hepatoma cells, an analogue of cAMP caused an elevation of PA activity partly due to induction of tPA mRNA and partly due to suppression of PAI-1 levels. Similar results were also found in the A15A5 cell line where cholera toxin an inducer of intracellular cAMP elevated tPA mRNA levels in the presence and absence of serum. Concurrently there was a suppression of PAI-1 message levels by CT under these conditions.

The promoters of human tPA and PAI-1 have 80% sequence homology to each other. The rat PAI-1 promoter has also been characterised and was found to share several promoter elements with the rat tPA gene (Johnson et al., 1992). DNase footprinting identified 8 protein binding sites in the rat PAI-1 promoter. Oligonucleotides for several known promoter sequences competed for 5 of the binding sites. The sites identified from the competitor experiments were one PEA3, one SP1, two sites with overlapping SP1 and NF1/CTF sequences and one NF1/CTF site. Two of the unidentified sites were found to be involved in transcriptional repression of the PAI-1 promoter. One of these sites has modest homology to

AP1 (86%) and CREB (75%) binding sequences. However Oligonucleotides for AP1 or CRE did not compete for this site indicating that these proteins do not bind to this site.

Two of the sites including one of the NF1/CTF sequences, were found to be located in a region that has 80% homology to the human PAI-1 promoter region containing signals for TGFß and glucocorticoid responsiveness. Another group (Bruzdzinski et al., 1990) has identifies several potential glucocorticoid response element sites within the rat PAI-1 promoter. One of these sites, at 1196 basepairs upstream from the TIS has 90% sequence homology to the GRE. It is likely that this glucocorticoid response element is responsible for the induction of PAI-1 in the A15A5 cell line.

The rat tPA gene does not have a glucocorticoid response element in its promoter, although it has a CRE response element. The differential regulation of tPA and PAI-1 by dexamethasone and cholera toxin is probably due to these differences in the promoters of these genes.

Although dexamethasone is proposed to modulate transcription of mRNA directly, it is also known to decrease stability of several transcripts. These include mRNA of c-myc (Maroder et al., 1990), interleukin-1ß (Lee et al., 1988), interferon ß (Peppel et al., 1991), uPA (Henderson and Kefford, 1993) and monocyte chemotactic-activating factor (Mukaida et al., 1991). A common denominator within these messages is that they all

contain repeats of the AUUUA pentamer in their 3' UTR.

Furthermore active protein synthesis was reported to be required for downregulation of stability by dexamethasone for all of the above transcripts except for interferon ß, implying that dexamethasone may be inducing a labile protein that target the destruction of these mRNA. Although dexamethasone mediated suppression of interferon ß mRNA stability was demonstrated to be through the AU rich region of its 3' UTR, the translation inhibitor cycloheximide which normally superinduces interferon ß mRNA, did not inhibit downregulation of the message by dexamethasone.

The transcriptional rate of both tPA and PAI-1 genes was found to be similar between A15A5 and C9 cell lines. Several of the transcription factor binding sequences found in the promoter of the PAI-1 gene are also present in the rat tPA promoter. There are three SP1 and one NF1/CTF sites in tPA. However, tPA also has a AP2 site and is thought to be a TATA-less promoter, whilst the PAI-1 has a TATAA sequence 30 bps upstream from the transcription initiation site. The tPA promoter has TTAAAA sequence at a similar position however it has been shown to be inactive in rat.

The differences in the promoter may be involved in the modulation of transcription of the genes whilst the similarities may relate to constitutive transcription of tPA and PAI-1. However basal levels are primarily regulated through post-

transcriptional mechanism for tPA.

These studies on the regulation of genes in the fibrinolytic system provides an insight in to coordinate regulation and expression of genes in a complex system. Another factor that has an important function in the fibrin/fibrinolytic system is the Tissue factor (TSF). This factor serves as the cellular receptor for clotting agent factor VII, which is the major initiator of coagulation in blood. Once bound to TSF, factor VII becomes rapidly activated (VIIa) initiating the blood clotting system (figure 8.1). The tissue factor also contain a 3' UTR AU-rich region. There are 4 repeats of the AUUUA pentamer within a region of 150 bps, 79% of which consist of A or U nucleotides. In the presence of cycloheximide TSF mRNA is superinduced transiently before becoming degraded rapidly (Ahern et al., 1993) and its stability was shown to be controlled through its AU element. Transfer of this AU region to the 3' UTR of ß globin reduces its half-life from greater than 20 hours to 2.4 hours, almost comparable to TSF half-life of 48 mins to 1.5 hours.

There is an interesting link between the regulation of TSF, PAI-1 and the inducers of fibrinolysis and ECM degradation. Several of the agents that are known to induce PAI-1 such as TNF, bacterial endotoxin and interleukin I also induces TSF mRNA expression. Dexamethasone which induces PAI-1 has been shown to suppress message levels of tPA, collagenase and

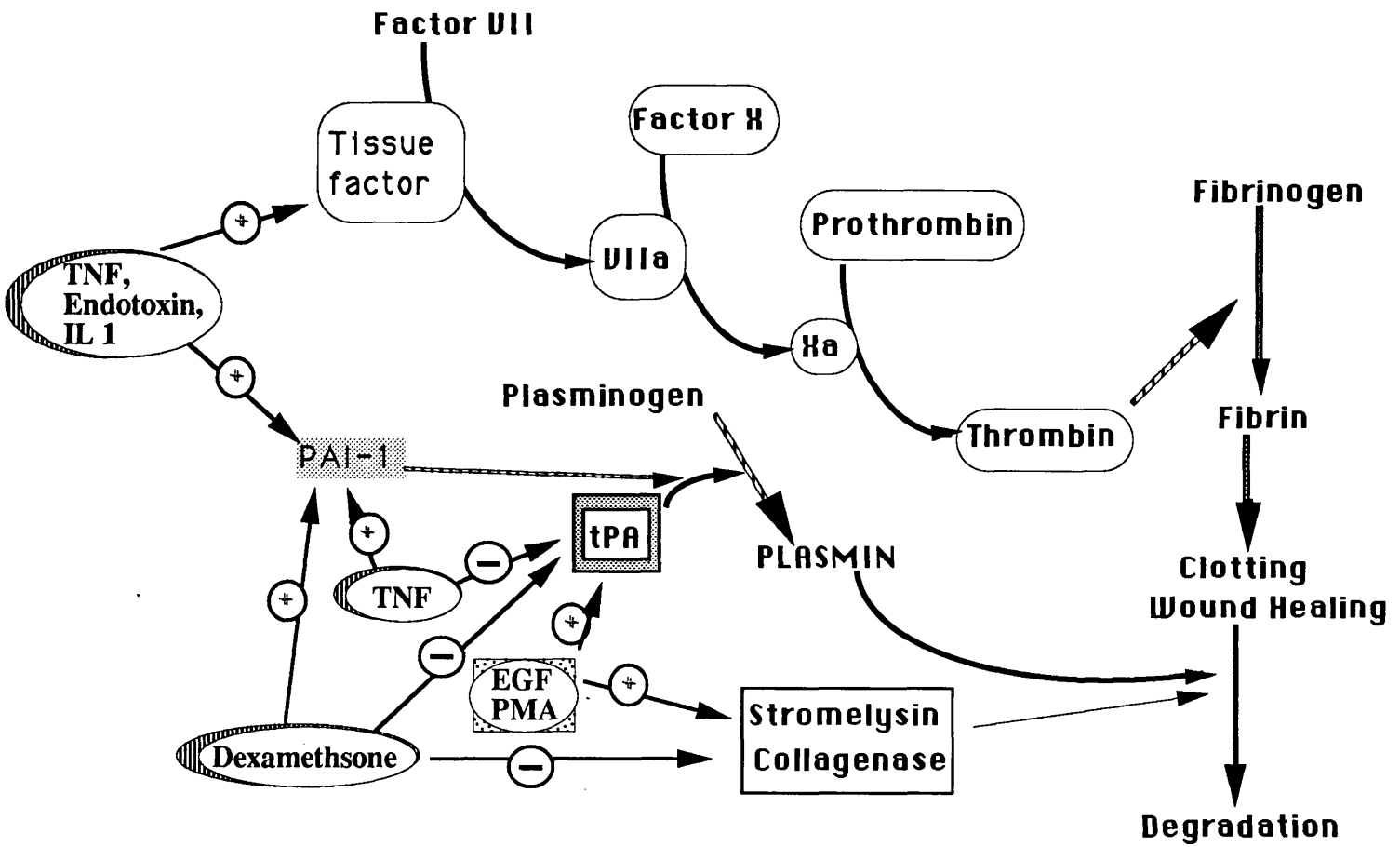


Figure 8.1 : The co-ordinate regulation of plasminogen activation and other related physiological events.

PA = plasminogen activator, STM= stromelysin, CLG= collagenase

stromelysin (Delany and Brinkerhoff, 1992). The human collagenase and stromelysin have 3 and 1 AUUUA pentamers respectively. Furthermore, PMA and EGF which induces tPA, collagenase and stromelysin mRNA, suppresses PAI-1 message (figure 8.1). This shows that there is a mechanism that coordinately up- or downregulated different components of the same system.

The coordinated regulation of mRNA levels may be confined to transcriptional processes, while the basal levels may be primarily controlled through post-transcriptional message stability, or both mechanisms may have a role. It is interesting that all of these transcripts (tPA, collagenase, stromelysin, PAI-1 and TSF) possess AREs in their 3'UTR which are supposed to target mRNA for destruction. However the information from all of these studies suggest that these elements may have a wider role in the regulation and expression of mRNA.

Whether the same factors are involved in regulating the constitutive mRNA levels of the opposing agents is not known. It is possible that cell may have evolved trans-acting factors with dual roles to coordinately regulate different sets of genes, that is, targeting one set of transcripts for destruction whilst protecting others from degradation.

Future directions:

1. The elevated tPA mRNA level does not fully account for the higher level (50 to 80-fold) of PA activity in the glioma cell line, which suggest that tPA mRNA is translated at a higher level in A15A5 than in ARBO C9. Sequences in the 5'UTR, particularly those that form secondary structures are important in regulating translation of mRNA (Pelletier and Sonenberg, 1987). Therefore it is important to sequence the 5'UTR of tPA from the cell lines that could identify differences in the translational activation of the message.

Also there are trans-acting factors that are involved in controlling message translation. The protein initiation factor 4E (eIF-4E) which recognizes the cap structure, "melt" 5' secondary structure and facilitate scanning for the initiation codon by the 40S subunit has an important regulatory role. Overexpression of eIF-4E has been shown to cause malignant transformation of NIH 3T3 cells (Lazaris-Karatzas et al., 1990).

2. To determine the cause of differential nuclear processing of the tPA and PAI-1 transcripts in A15A5-

- sequence the 3'UTR of PAI-1 from the cell lines A15A5 and ARBO C9
- identify whether the AU-rich elements within the UTR of the two transcripts have a distinct role in the regulation of stability.
- characterise possible nuclear trans-acting factors that associate with the 3'UTR of the two messages to define whether they are same or different.

Appendix A

The sequence of the Rat tPA cDNA fragment sequenced. As the sequences from A15A5 and ARBO C9 were identical, only one sequence is shown. The numerical figures correspond to positions in the published sequence by Feng et al. (1990). The AATAAA polyA addition site and the proposed AU-rich protein binding sites are highlighted and underlined respectively.

```
     Ile Leu  Ile Gly  Lys  Thr Tyr Thr Ala  Trp     Arg Ala  Asn  Ser Gln
811  ATC CTG  ATA GGC  AAG  ACT TAC ACA GCG  TGG     AGG GCC  AAC  GCA CTT

Ala  Leu Gly  Leu Gly      Arg  His  Asn  Tyr Cys  Arg  Asn  Pro  Asp  Gly
GGC  TCC CAG  CTG GGC      AGA  CAC  AAT  TAT TGC  CGG  AAC  CCA  GAT  GGG

Asp  Ala Lys  Pro Trp  Cys  His  Val  Met  Lys      Asp  Arg  Lys  Leu  Thr  Trp
GAT  GCC AAA  CCT TGG  TGC  CAC  GTG  ATG  AAG      GAC  CGA  AAG  CTG  ACA  TGG

Glu  Tyr Cys  Asp        Met  Ser  Pro  Cys  Ser  Thr  Cys  Gly  Leu  Arg  Gln
GAA  TAT TGC  GAC (961)  ATG  TCC  CCA  TGC  TCC  ACC  TGC  GGC  CTG  AGG  CAA

Tyr  Lys Gln  Pro Gln  Phe  Arg  Ile  Lys  Gly      Gly  Leu  Phe  Thr  Asp
TAC  AAA CAG  CCT CAG  TTT  CGA  ATT  AAA  GGA      GGA  CTC  TTC  ACA  GAC

Ile  Thr Ser  His Pro      Trp  Gln  Ala  Ala  Ile  Phe  Val  Lys  Asn  Lys
ATC  ACC TCA  CAC CCT      TGG  CAG  GCC  GCC  ATC  TTT  GTC  AAG  AAC  AAG

Arg  Ser  Pro  Gly  Glu  Arg  Phe  Leu  Cys  Gly          Gly  Val
AGG  TCT  CCA  GGA  GAG  AGA  TTC  CTG  TGT  GGA (1114)  GGG  GTG

Leu  Ile  Ser  Ser  Cys  Trp  Val  Leu      Ser  Ala  Ala  His  Cys  Phe
CTG  ATC  AGT  TCC  TGC  TGG  GTG  CTA      TCT  GCC  GCC  CAC  TGC  TTT

Val  Glu  Arg  Phe      Pro  Pro  His  His  Leu  Lys  Val  Val  Leu  Gly
GTA  GAG  AGG  TTT      CCA  CCC  CAT  CAT  CTT  AAA  GTG  GTC  TTG  GGC

Arg  Thr  Tyr  Arg  Val  Val  Pro  Gly  Glu  Glu      Glu  Gln  Thr  Phe  Glu
AGA  ACA  TAC  AGA  GTG  GTC  CCT  GGA  GAG  GAG      GAG  CAG  ACA  TTC  GAG

Ile  Glu  Lys  Tyr  Ile          Val  His  Lys  Glu  Phe  Asp  Asp  Asp  Thr  Tyr
ATC  GAA  AAG  TAC  ATA (1264)  GTC  CAT  AAG  GAA  TTT  GAT  GAC  GAC  ACT  TAT

Asp  Asn  Asp  Ile  Ala  Leu  Leu  Gln  Leu  Arg      Ser  Asp  Ser  Ser  Gly
GAC  AAT  GAC  ATC  GCA  TTA  CTG  CAG  CTG  AGG      TCA  GAT  TCC  AGT  CAG

Cys  Ala  Gln  Glu  Ser      Ser  Ser  Val  Gly  Thr  Ala  Cys  Leu  Pro  Asp
TGT  GCC  CAG  GAG  AGC      AGT  TCT  GTC  GGC  ACT  GCC  TGC  CTC  CCT  GAC

Pro  Asp  Val  Gln  Leu  Pro  Asp  Trp  Thr  Glu          Cys  Glu  Leu  Ser  Gly
CCC  GAC  GTA  CAG  CTC  CCT  GAC  TGG  ACA  GAG (1414)  TGT  GAG  CTT  TCT  GGC
```

Tyr Gly Cys His Glu Ala Ser Ser Pro Phe Phe Ser Asp Arg Leu
TAC GGC AAG CAT GAG GCA TCC TCT CCT TTC TTC TCT GAC CGG CTG

Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser
AAG GAG GCT CAC GTC AGA CTG TAT CCG TCC AGC CGC TGT ACC TCA

Gln His Leu Phe Asn Lys Thr Ile Thr Ser Asn Met Leu Cys Ala
CAG CAT CTG TTT AAC AAA ACC ATC ACG AGC AAC ATG CTG TGT GCA

Gly Asp Thr Arg Thr Gly Gly Asn Gln Asp Val His Asp Ala
GGA GAC ACC CGA ACT GGG GGC AAC CAA GAC (1564) GTC CAT GAC GCG

Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Met Ile Asp Lys Arg
TGC CAG GGT GAC TCA GGA GGC CCT GTG GTG TGC ATG ATC GAT AAG CGG

Asp Val Pro Gly Ile Tyr Thr Lys Val Thr Asn Tyr Leu Asn
GAC GTG CCA GGG (1714) ATA TAC ACA AAG GTC ACT AAT TAC CTG AAC

Trp Ile Gln Asp Asn Met Lys Gln
TGG ATC CAA GAC AAC ATG AAG CAA (1767) TGA CAA AGAAAGCCCA

GCTCCTTAAA CCCAGAGGAC CTGCCTTCCT CTTCCACAGA AGATACGCCT GAAAGGCCAA

GCGTTCTTCG (1853) CAGGCTCGTC CTCCTGAGCT GCCGCTCAGC AGAGGGAGTG

ACAGTCTTTA GGCACAGACA GCGTTTACTT TGTGACAGGT ACTTCACAAA CTTGTACGTT

TTAAGGGTGA AGGTCTGACT TTAGAATCAG TTCTGTCAGA TGAGATGACA (2003)

GGGAAATGCC AACCTTCCTA TAACTCTAAG ATTTTAAAAA GAGAAGTAGA CCAAAGTCCA

CCCTTCCTGG ACCACTATTT TGTACACTGA ACCACAAGAT CGTGTCTCAA CAGTGAAATA

CAACTTGATC TTTCAGGAGT AAAAGTCTGC (2153) ACTGAGGACA AGAATGTGTT

TTTATAGTTA CACAGGGGCC CAGCATGGCC TCCAAGAGAA GGAAGGGGTT AGCTGATCAG

ACCACAGCCC CCTAAAACCC TTGAAATCAA ATATTCCCCA TCCTCCCACA ATCCTCAACT

CTTGGGGCAT ATCCCTTTGT ACACAGTGTA GATGTCTTTT TCTTTATAAA CTTTCCAGAT

GGCTGGGAGA ACTGTATGAT TTTAATATTC GATGAAATGA CACTAGTAT ATTTA TATTTG

AATCT ATTTA GTTTTTACTG TGTTACTAGA ACTCTGTATT ATGCTGTACT

GAAAT **AATAAA** TTCCAAGGT ATTTTTCACA CTTTTAAA (2503) AAA

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Addendum.
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I would like to thank Dr. Joan Roscoe for giving me the opportunity to carry out this project, for her guidance throughout the years and for the generosity of her time during the writing-up of the work.

I like to thank Ms. Rosemary Zammit-Pace for the collaborative work, expertise in maintaining cell cultures and for help with general laboratory duties.

Furthermore I am grateful to Professor David Latchman for the use of facilities in the Department of Medical Molecular Biology and to Dr. B.Planskoy for providing access to the Joyce Lobel Densitometer.

I am also grateful to my friends Ms Janet Cooper and Ms Mary Ferdinand for their help with the typing of this project.

Finally I would like to thank my parents for all the support and encouragement they have given me throughout the years.