MANIPULATION OF GENE EXPRESSION IN EARLY MOUSE EMBRYO

by

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## ABSTRACT

Maternal (oocyte) and embryonic programmes of hypoxanthine phosphoribosyl transferase (HPRT) and adenine phosphoribosyl transferase (APRT) gene expression have been investigated in mouse oocytes and during preimplantation development. The onset of the embryonic HPRT gene occurs following fertilization, or parthenogenetic activation, before the 4-cell stage. The oocyte-determined increase in HPRT activity due to preformed mRNA continues in aging oocytes, as well as in fertilized or activated eggs, i.e. irrespective of the initiation of embryonic development. Attempts were made to determine whether there is a detectable time difference in the onset of maternal and paternal genomes by assaying the onset of embryo- coded HPRT activity in embryos of different maternal and paternal X-chromosome constitution, but due to difficulties in comparable staging of embryos, no definite conclusion could be drawn.

The expression of an exogenously introduced HPRT minigene has been monitored throughout preimplantation development. The embryos injected with supercoiled HPRT minigene showed an approximately twofold increase in HPRT activity at the 2-cell stage compared with control uninjected embryos. Linear minigene DNA was less efficient in giving active enzyme. The efficacy of three different promoters were studied in 2-cell mouse embryos using the expression of the HPRT minigene as a reporter function. The mouse HPRT promoter and the uninduced mouse metallothionein-1 (MT-1) promoter functioned equally well whereas the viral SV40 promoter did not allow HPRT expression. The mouse MT-1 promoter linked to the HPRT minigene allowed induction of HPRT gene expression in mouse embryos cultured in the presence of

cadmium.

The inhibition of enzyme expression from injected minigene DNA is mediated by simultaneous injection of a fivefold molar excess of HPRT antisense DNA. The same negation of exogenous HPRT activity was observed with simultaneous injection of HPRT exon-1 antisense DNA. The use of an inducible HPRT antisense construct achieved repression of gene activity with equivalent molar amounts of antisense to the sense molecules.

Transgenic mice were produced with an antisense HPRT minigene construct attached to the inducible mouse MT-1 promoter. The prospect of "cancelling" the endogenous "sense" gene activity in these mice at a specific stage of development by applying the induction stimulus is discussed.

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# CHAPTER 1

## MATERNAL AND EMBRYONIC PROGRAMMES OF HPRT AND APRT EXPRESSION IN OOCYTES AND PREIMPLANTATION EMBRYOS

## 1.1 INTRODUCTION

### 1.1.1 Early mouse embryo development

Embryonic development is initiated by fertilization. The stages of preimplantation and post-implantation development up to the tenth day of gestation are diagrammed in figure 1.1. The first cleavage division occurs about 24 hours after fertilization and the second and third cleavages, which are not entirely synchronous, follow at intervals of about 12 hours. At the early 8-cell stage the embryo acquires a spherical shape in a process called compaction. The embryo is called a morula from the compacted 8-cell to the 16- to 32-cell stage. At about 3 days after fertilization the embryo moves from the oviduct to the uterus. The embryo starts to cavitate in the uterus, the fluid filled cavity (blastocoel) expands, and the blastocyst is formed. At this stage, two cell types are differentiated; the inner cell mass (ICM) and the outer sphere of cells, the trophectoderm. The ICM cells proliferate and a layer of primary endoderm differentiates on the blastocoelic surface of the ICM. The trophectoderm becomes divided into a polar component, which overlies the ICM, and a mural component which makes up the remainder. The polar trophectoderm cells continue to proliferate while the mural trophectoderm cells become transformed into polyploid giant cells in which DNA replication continues (endoreplication) but without mitosis.

At about this stage the embryo hatches from its non-cellular shell (the zona pellucida) and becomes implanted in a uterine crypt. It is only after implantation, that differentiation and organization

**Figure 1.1** Schematic diagram of the development of the mouse embryo from fertilized egg to the 10th day of gestation.

d-days post-fertilization; PB-polar bodies; PN-pronucleus; B-blastomere; ICM-inner cell mass; T-trophectoderm; Ep-epiblast; Eex-extraembryonic ectoderm; GC-trophoblast giant cells; VEex-extraembryonic visceral endoderm; VEem-embryonic visceral endoderm; PE-parietal endoderm; EPC-ectoplacental cone; Ch-chorion; EX-exocoelom; YC-yolk cavity; M-mesoderm; AC-amniotic cavity; HP-head process; PS-primitive streak; Am-amnion; Ne-neurectoderm; G-gut; H-heart; No-notochord; HF-head fold; S-somite; OA-omphalomesenteric artery; Al-allantois; VYS-visceral yolk sac; FLB-forelimb bud; Ot-otic capsule; Op-optic evagination; SE-surface ectoderm.

Figure 1.1

1c

5-8c

Blastocyst

5.5d

6.5d

7.5d

8.5d

9.5d
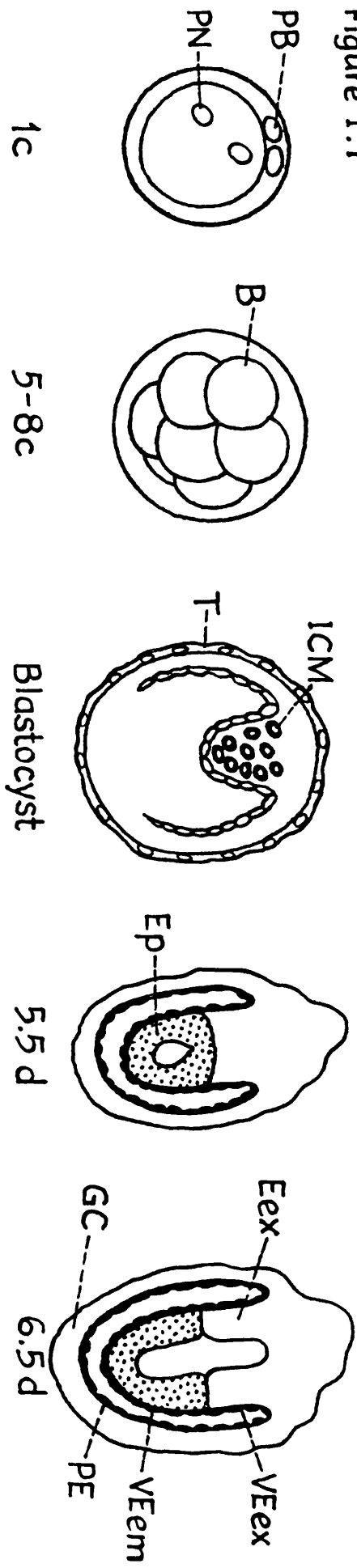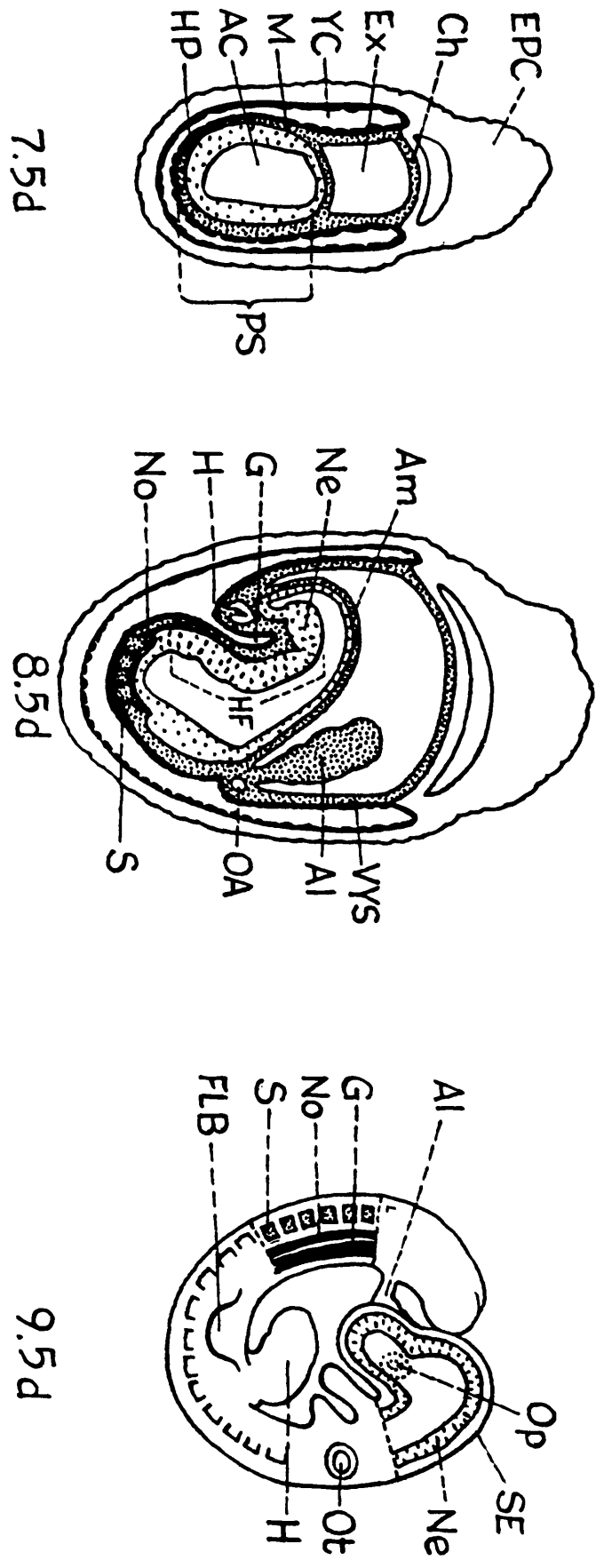
of the fetus occurs. The primary endoderm gives rise to both parietal endoderm and visceral endoderm around the egg cylinder. This layer of cells are characterised by the synthesis of α-fetoprotein and transferrin (Dziadek, 1979). The egg cylinder consists of epiblast (derived from the ICM) and extraembryonic ectoderm derived from the polar trophectoderm. The polar trophectoderm also forms the ectoplacental cone and, as it proliferates, it produces further layers of giant cells which move around and reinforce the trophoblast.

At about 6½-7 days after fertilization the primitive streak appears, marking the onset of axis formation and bilateral symmetry in the embryo. The earliest germ cells have been detected on day 7 at the base of allantois (Ginsberg et al, 1989). At this stage the embryos have a head fold and a tail bud with appearance of the first somites soon to follow. Though gastrulation persists up to and during the 10th day of gestation, towards the end of the 8th day the first signs of organogenesis are apparent.

## 1.1.2 Early developmental programmes

In many species, including the mouse egg, the early events that take place following fertilization are largely under maternal control (reviewed by Davidson, 1976; Johnson, 1981; Woodland et al, 1979; Rosenthal et al, 1980). In the ovulated mouse egg, the bulk of the RNA is made during the growth phase of the oocyte and inherited along with the ribosomal proteins (Bachvarova & De Leon, 1980; Young et al, 1978; LaMarca & Wassarman, 1979). From the total RNA in the ovulated egg, about 23-25 pg (7-8% of total RNA) is poly A+ RNA, sufficient to

contribute substantially to early cleavage protein synthesis
(Bachvarova & De Leon, 1980).

Braude et al (1979) found a group of proteins  appearing at the
2-cell stage on two dimentional gels.  Inhibition of  mRNA synthesis
by $\alpha$-amanitin did not prevent synthesis of these proteins, even when
the eggs were fertilized in presence of the inhibitor (Flach et al,
1982).  It may be concluded that these proteins are translated from
stable mRNA inherited in the egg cytoplasm.  In addition, Monk and
Harper (1983) have presented evidence for the synthesis of a specific
enzyme, the X-coded HPRT (hypoxanthine phosphoribosyl transferase), on
stable maternal mRNA up to the 8-cell stage.  The events determined by
macromolecules inherited in the egg cytoplasm will be termed "the
oocyte programme".

The initiation of embryogenesis may involve the following two
programmes: (i) the oocyte propramme and, (ii) the activation
programme.  The activation programme, whether initiated by
fertilization or parthenogenetic activation will include activation of
stored, inactive (masked) maternally-inherited macromolecules, as well
as the activation of transcription of the embryo's own genes.  Mouse
eggs may be stimulated to develop parthenogenetically (haploid or
diploid) in culture (Niemerko, 1975; Kaufman et al, 1977; Markert,
1982; Kaufman, 1982).  The embryos will undergo normal preimplantation
development but die in the early postimplantation stages (Witkowska,
1973a; Witkowska, 1973b; Kaufman et al, 1977; Kaufman, 1978).

The role of maternally-inherited macromolecules and initiation, or onset, of embryonic transcription in the mouse is reviewed by Johnson (1984). The oocyte programme may be activated by the effect of luteinizing hormone on the granulosa cells of the cumulus mass. The breakdown of the germinal vesicle then follows resulting in release of proteins previously secluded in the nucleus (Wassarman et al, 1978). The release of these proteins initiates many morphological and molecular changes in the oocyte (Van Blerkom & Runner, 1984). Warnes et al (1977) have shown that the nuclear events of oocyte maturation in sheep can be induced without bringing about the cytoplasmic changes, but these oocytes are not viable (Moor & Trounson, 1977). The above findings suggest that interaction of cytoplasmic and nuclear events may be required.

Fertilization (or parthenogenetic activation) initiates activation of a new synthetic programme in the zygote. However, if fertilization does not occur, the egg still continues with some of the changing qualitative pattern of biosynthetic activity (Howlett & Bolton, 1985). A comparision of polypeptides synthesized by developing embryos and by the aging oocyte shows that some of these processes take place irrespective of fertilization. The oocyte programme may be proceeding independently of fertilization. The activation programme, initiated by parthenogenetic activation or sperm penetration, would then be superimposed on the oocyte programme. Many newly synthesized polypeptides are common to fertilized eggs and aging oocytes.

The contribution of the oocyte programme to the developing embryo will be ended when maternally-inherited macromolecules are diluted out or destroyed. There is a loss of maternally-derived mRNA from the embryo by the late 2-cell stage. This is evidenced by the cessation of synthesis of proteins on stored mRNA ($\alpha$-amanitin insensitive protein synthesis) (Flach et al, 1982; Bolton et al, 1984) and the decline in total polyadenylated RNA (Piko & Clegg, 1982; Clegg & Piko, 1983a,b). A recent report by Paynton et al (1988) suggests significant loss of maternal message for actin, HPRT and tubulin by the late 2-cell stage. The loss of some or all maternal transcripts may occur even earlier than 2-cell stage (Piko et al, 1984). However, maternally-inherited enzymes appear to be more stable, some may be degraded from the 8-cell stage (reviewed by Harper and Monk, 1983), others might persist beyond this stage (see later).

The analysis of macromolecular synthesis in preimplantation mouse embryos suggests that the activation (Knowland and Graham, 1972) and expression of the embryonic genome may occur as early as 2-cell stage (Young et al, 1978; Levey et al, 1978; Braude et al, 1979; Flach et al, 1982). Some RNA, heterogenous in size, is synthesized at the 2-cell stage and as some of this new RNA is polyadenylated, presumably it is mRNA (Levey et al, 1978). Clegg and Piko (1977) detected $^3$H-uridine incorporation into RNA even at the 1-cell stage. Other investigations suggest the activation of certain embryonic genes may occur after the 2-cell stage. The paternally-inherited isozyme of GPI (glucose phosphate isomerase) and embryonic HPRT activity can be detected at the 8-cell stage (Brinster, 1973; Harper & Monk, 1983) and $\beta$-glucuronidase at the morula stage (Wudl & Chapman, 1976).

The experiments reported in this chapter are designed to investigate the oocyte and activation programmes with respect to HPRT and APRT expression, viz,

(i) the increase in HPRT activity due to translation of stored maternal mRNA and the onset of embryonic HPRT gene expression in preimplantation mouse development (reproducing and extending some of the earlier investigations of Harper & Monk, 1983)

(ii) whether the same maternal- and embryo-coded increases of HPRT activity occur in parthenogenetically activated embryos

(iii) whether the maternal mRNA (for HPRT) is translated in the absence of fertilization or parthenogenetic activation in oocytes

(iv) whether HPRT gene transcription occurs in aging oocytes

(v) whether similar profiles occur for autosome-linked adenine phosphoribosyl transferase (APRT) in unfertilized, fertilized or parthenogenetically activated eggs.

The increase in HPRT and APRT activities were studied throughout preimplantation development of fertilized and parthenogenetic embryos and in aging unfertilized eggs. New mRNA synthesis was inhibited by culturing the embryos in 10 µg/ml of α-amanitin which specifically blocks RNA polymerase II (Lindell et al, 1970; Levey & Brinster, 1978). The effects of this drug on both HPRT and APRT activities were monitored. The results reported in this chapter reveal some

interesting changes at the molecular level during early embryogenesis. On the bases of these findings the later chapters (chapter 2-4) of this thesis deal with manipulation of endogenous and exogenous HPRT gene expression in early cleavage stages of mouse preimplantation development.

## 1.2 MATERIALS AND METHODS

### 1.2.1 Embryo collection

The eggs were obtained from 3-5 weeks old F1 female (CBA x C57BL) mice. The mice were superovulated by intraperitoneal injection of 5 i.u. of pregnant mare serum (PMS, Intervet) followed 45-48 hours later by 5 i.u. of human chorionic gonadotrophin (hCG, Intervet). They were then mated with F1 males to produce fertilized eggs. To obtain parthenogenetic and unfertilized eggs, the females (after administering hCG) were kept separately. Ovulation was assumed to occur 12 hours after the hCG injection (Gates & Beatty, 1954), and the presence of a vaginal plug was taken as proof of mating. The age of the embryo is normally expressed as "hours post hCG" (h.p.hCG).

On the first day of pregnancy, one cell zygotes were isolated from the oviducts of mated females and were separated from cumulus cells by 5-7 minutes incubation at room temperature in hyaluronidase (300 i.u/ml, Sigma). The eggs were then washed free of enzyme in PB1 medium (Whittingham & Wales, 1969). The fertilized eggs were cultured (see below) for an hour to equilibrate before harvesting the first set of samples. To harvest, only healthy appearing eggs and embryos were collected either singly or in groups. The embryos were first washed in PB1.PVP (PB1 with 4 mg/ml polyvinyl pyrrolidone (PVP) instead of albumin) and transferred in 5 μl of the same medium to 10 μl Drummond microcaps (Scientific Supplies). The ends of the microcaps were sealed by melting the glass in a flame and the samples stored at

-70°C. Supernatant extracts were prepared by freeze-thawing three times followed by centrifugation for 10 minutes.

## 1.2.2 Parthenogenetic embryos

To obtain diploid parthenogenetic embryos, unfertilized eggs were activated parthenogenetically following a brief exposure (7 minutes) to 7% ethanol in medium 16 (M16, Whittingham, 1971) at 37°C. After two washes through M16 the activated eggs were transferred to 25 µg/ml cytochalasin B (Sigma) prepared in medium 16 to suppress the formation of the second polar body. After incubating 4 hours at 37°C, the eggs were then washed through four droplets of medium 16. The diploid parthenogenetic embryos obtained were verified by the presence of a single polar body. The parthenogenotes were cultured and harvested for assay at intervals.

## 1.2.3 Unfertilized eggs

The 1-cell eggs were released from the oviducts of the unmated females after 19-20 hours post hCG and separated from the cumulus cells as described above. Samples of unfertilized eggs were harvested after various periods of culture.

## 1.2.4 Embryo culture

Droplets of medium 16 with or without the inhibitor of RNA synthesis ($\alpha$-amanitin, see below) were prepared under paraffin oil (BDH) in plastic culture dishes and equilibrated in a humidified 5%

$CO_2$-in-air atmosphere at $37^{\circ}C$ for about an hour. Embryos from a number of litters were usually pooled and added to a culture droplet, via a wash droplet, in groups of between 20 to 30. The cultured embryos were harvested after washing in PB1.PVP to remove traces of oil and medium 16. Both experimental (plus RNA synthesis inhibitor) and control (minus RNA synthesis inhibitor) embryos were harvested at the 2-cell, 4- to 8-cell and morula stages after 1, 2 and 3 days of culture respectively.

## 1.2.5 $\alpha$-amanitin treatment

A stock solution of RNA synthesis inhibitor, $\alpha$-amanitin (Sigma, 1 mg/ml in medium 16) was aliquoted in 10 $\mu$l volumes and the aliquots stored at $-70^{\circ}C$. Embryos were cultured in a final concentration of 10 $\mu$g/ml of $\alpha$-amanitin for specified times and harvested. Control embryos incubated without $\alpha$-amanitin were harvested at the same time for enzyme assay.

## 1.2.6 Embryo and liver extracts

F1 female mice (CBA x C57) were mated with F1 males. Mating is confirmed by the presence of a vaginal plug on the first day of pregnancy, this being gestation day $\frac{1}{2}$. On $3\frac{1}{2}$ days the blastocysts were collected from females killed by cervical dislocation. Individual uterine horns were dissected away from the mesentery and cut at the uterotubal junction and cervix. A 25-gauge needle attached to a syringe was inserted into the lumen of each uterine horn at the ovarian end and 0.5 - 1 ml of PB1 medium (Whittingham & Wales, 1969)

flushed through into a glass cavity block. This procedure was repeated by inserting the needle from the cervical end to make sure all the blastocysts have been flushed out. Blastocysts were collected and washed in PB1.PVP medium and transferred in groups of 20-30 in 5 µl of the same medium to 10 µl microcaps. Any subsequent dilutions were made in PB1.PVP medium. The liver extracts were made by cutting the mouse liver into fine pieces with sharp dissecting scissors and sieving through a muslin cloth. The liver extract was diluted 1:200 in PB1.PVP medium for HPRT and APRT assay.

### 1.2.7 HPRT and APRT enzyme assay

HPRT and APRT activities were measured simultaneously by incubating embryo extracts in a 50 µl reaction mixture at 37°C as described by Monk (1987). The final concentration of reaction mixture consists of sodium phosphate buffer (38.75 mM, pH 7.4), magnesium chloride (5 mM), PRPP (1 mM), $^3$H-hypoxanthine (10 µM, specific activity 1625 Ci/M, Amersham) and $^{14}$C-adenine (10 µM, specific activity 270 Ci/M, Amersham). For maximum sensitivity, undiluted uniformly-labelled $^{14}$C-adenine at the highest specific activity available was used. Care must be taken to avoid any kind of contamination through handling microcaps, pasteur pipettes and reaction tubes, and all the components of the reaction mixture should be kept sterile at 4°C. The preparation of stock solutions is shown in table 1.1.

The enzyme assay was carried out by incubating embryo extract in a 50 µl reaction mixture at 37°C in a water bath. The reactions were

**Figure 1.2** Schematic diagram showing double microassay for HPRT and

APRT activities in an embryo extract

For detail see text

The diagram was kindly provided by Dr.Marilyn Monk

Figure 1.2

Freeze thaw and spin

5μL supernatant

$$\left[\begin{array}{l} [^3\text{H}] \text{ hypoxanthine} + \text{PRPP} \\ [^{14}\text{C}] \text{ adenine } + \text{ PRPP} \end{array}\right.$$

X-coded HPRT
$\longrightarrow$
$[^3\text{H}]$ IMP (or GMP)

APRT autosome-coded (chromosome 8)
$\longrightarrow$
$[^{14}\text{C}]$ AMP

$\left.\begin{array}{l} \\ \end{array}\right\}$ LaCl$_3$ $\longrightarrow$

filter and count
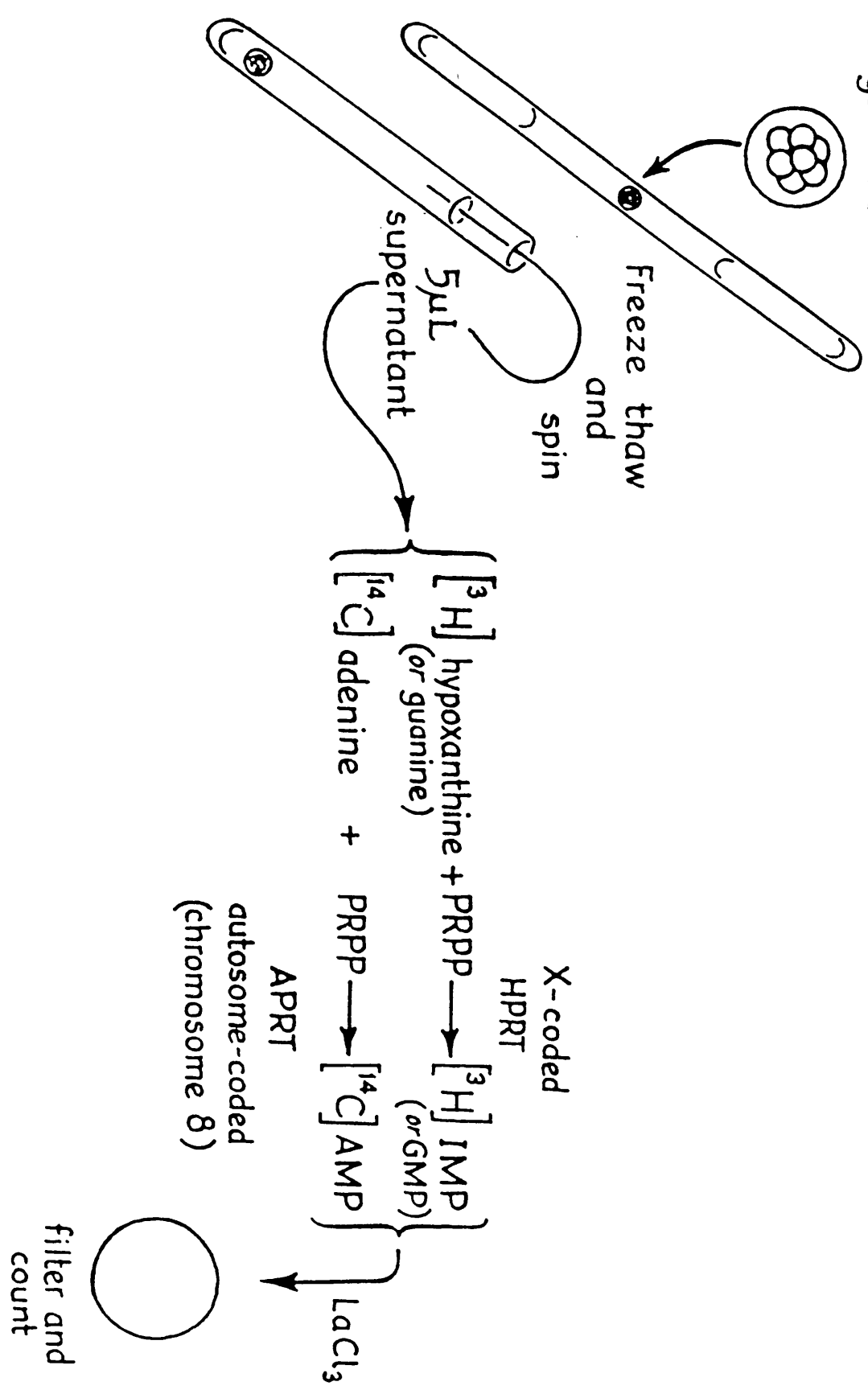
**Table 1.1 - Preparation of reaction mixture for HPRT and APRT assay**

<u>STOCK SOLUTIONS</u>:

1. $MgCl_2.6H_2O$ - Magnesium chloride, 50 mM in distilled water, filter sterilized, aliquoted and stored at $4^{\circ}C$.

2. PRPP - Phosphoribosyl pyrophosphate, 10 mM prepared fresh in sterile water.

3. $NaPO_4$ - 50 mM sodium phosphate buffer (pH 7.4), prepared by mixing
1.75 ml of .2 M $NaH_2PO_4.2H_2O$, 8.25 ml of .2 M $Na_2HPO_4.12H_2O$ and 30 ml of water, filter sterilized, aliquoted and stored at $4^{\circ}C$.

4. **Hypoxanthine** - 12.9 μM cold (unlabelled) hypoxanthine prepared with 50 mM $PO_4$ buffer, filter sterilized, aliquoted and stored at $4^{\circ}C$.

5. $^3H$-hypoxanthine - (TRA.74, specific activity 1625 Ci/M) - 12.9 μM in $PO_4$ buffer, pH 7.4, filter sterilized, aliquoted and stored at $4^{\circ}C$. On the day of assay, this stock solution was diluted as required with 12.9 μM unlabelled hypoxanthine in $PO_4$ buffer (see above) to obtain the desired specific activity.

6. $^{14}C$-adenine - [U-$^{14}C$]adenine (CFA 436, specific activity 270 Ci/M) as supplied. Molarity varies between batches so the appropriate volume is added to the reaction mixture to obtain a final concentration of 10 μM.

REACTION MIXTURE

| Component | Relative volume | Final concentration |
|---|---|---|
| $MgCl_2$ | 5 μl | 5 mM |
| PRPP | 5 μl | 1 mM |
| $PO_4$ buffer, with $^3H$-hypoxanthine | 38.75 μl | 38.75 mM / 10 μM |
| $^{14}C$-adenine | 1-3 μl | 10 μM |

incubated for 3 hours and terminated by the addition of 1 ml of ice-cold lanthanum chloride (100 mM) which contained excess unlabelled hypoxanthine (1 mM) and adenine (1 mM). The tubes were kept on ice until filtering. The lanthanum chloride-precipitated IMP and AMP products, were collected by filtration onto numbered 2.4 cm glass fibre filters (Whatman) and the sample washed thoroughly three times with 10 ml distilled water. The efficiency of washing was monitored by the reproducibility of replicate blank samples (with PB1.PVB medium minus extract). The filters were dried in a hot air oven.

The dry filters were placed in plastic scintillation inserts with 4 ml scintillation fluid (Liquiflor, New England Nuclear) and counted on a LKB  scintillation counter on a dual label counting programme. To work out the spill over of $^3$H counts into the $^{14}$C channel and vice versa, the reaction mixtures with $^3$H-hypoxanthine alone (water instead of $^{14}$C-adenine) and $^{14}$C-adenine alone (PO$_4$ buffer instead of labelled $^3$H-hypoxanthine) with experimental samples were incubated and processed in the usual way. Two blank reaction mixtures (with single labelled substrates and with PB1.PVP medium) were also incubated to substract from the values obtained with the samples. To determine the efficiency of counting of $^3$H and $^{14}$C, duplicate 5 µl volumes of each reaction mixture were spotted onto dry clean filters and counted as above. Knowing the number of µCi of $^3$H and $^{14}$C in the 5 µl reaction mix, and given that 1 µCi produces $2.2 \times 10^6$ dpm (disintegrations per minute), the cpm (counts per minute) obtained gives the eoc (efficiency of counting) under the conditions of the experiment and with the dual label counting programme used.

## 1.3    RESULTS

### 1.3.1  The enzyme pathways

The reactions catalised by HPRT and APRT are shown in figure 1.2. The diagram shows the assay of a single embryo extract under the conditions described in section 1.2.6. The microcaps containing the embryo in 5 $\mu$l PB1.PVP medium are heat sealed at the ends and stored at -70oC. For the assay, the sample is freeze-thawed three times, centrifuged and the supernatant is added to the reaction mixture for assay. The final products (IMP and AMP) are then collected on a glass fibre filter and counted in a scintillation counter.

The assay has been in use for over ten years and its sensitivity, reliability, and accuracy well established. The validity of the microassay was shown by independently identifying the substrates and the products by thin layer chromatography (Harper, 1981). The linearity of enzyme activity has been tested with concentration of enzyme and time of incubation (Monk & Kathuria, 1977). To achieve greater sensitivity, the enzyme reaction can be incubated for long periods provided there is no risk of contamination and the activity of each enzyme remains linear with time (see Monk, 1987).

The assay is sensitive enough to measure both HPRT and APRT activities in a single blastomere of a cleavage-stage embryo. It has been used to develop procedures for preimplantation diagnosis (Monk et al, 1987; 1988). HPRT-deficient male embryos could be diagnosed by assay of single blastomeres biopsied from 8-cell embryos derived from

a heterozygous HPRT+/HPRT- carrier mother. The sex of an embryo could also be accurately diagnosed by assay of HPRT dosage in a biopsied blastomere (Monk & Handyside, 1988).

In the experiments reported in this thesis some variability in enzyme activity is observed from experiment to experiment. A preliminary investigation was made to discover the source of variability. The reaction mixture is prepared from previously made stock solutions (see table 1.1) except for PRPP, which is prepared just prior to the assay. An experiment was designed to test the effect of different concentrations of PRPP in liver and embryo extracts on HPRT and APRT activities.

## Effect of PRPP concentration on HPRT and APRT activities

The HPRT and APRT assay was carried out as described in section 1.2.6. Figure 1.3 shows the product counts of $^3$H-IMP and $^{14}$C-AMP resulting from incubation of blasotocyst extracts and of a 1 in 200 dilution of liver extract for 3 hours with varying concentrations of PRPP (0.1, 0.5, 1, 5, 10 and 20 mM). With PRPP concentrations over 0.5 mM the product counts of both $^3$H-IMP and $^{14}$C-AMP were decreased in both liver and blastocyst extracts (figure 1.3). The HPRT and APRT activities in liver extracts were maximum at 1 mM of PRPP (table 1.2), however, concentrations below 1 mM were not tested with liver extracts. Similar results were obtained with blatocyst extracts where 0.5 and 1 mM PRPP concentrations resulted in maximum enzyme activity (table 1.2). The results show that PRPP concentration is very critical for enzyme activity and that higher concentrations of PRPP

**Figuere 1.3** Effect of PRPP concentrations on product counts of $^3$H-IMP

and $^{14}$C-AMP in liver and blastocyst extracts

Liver extracts; HPRT = O-O, APRT = ●-●

Replicate samples were assayed for each PRPP concentration to

calculate the SE

Blastocyst extracts (equivalent to extract of 2

blastocysts/sample); HPRT = △-△, APRT = ▲-▲

Only two replicate samples were assayed for each PRPP

concentration therefore the SE could not be determined

Figure 1.3
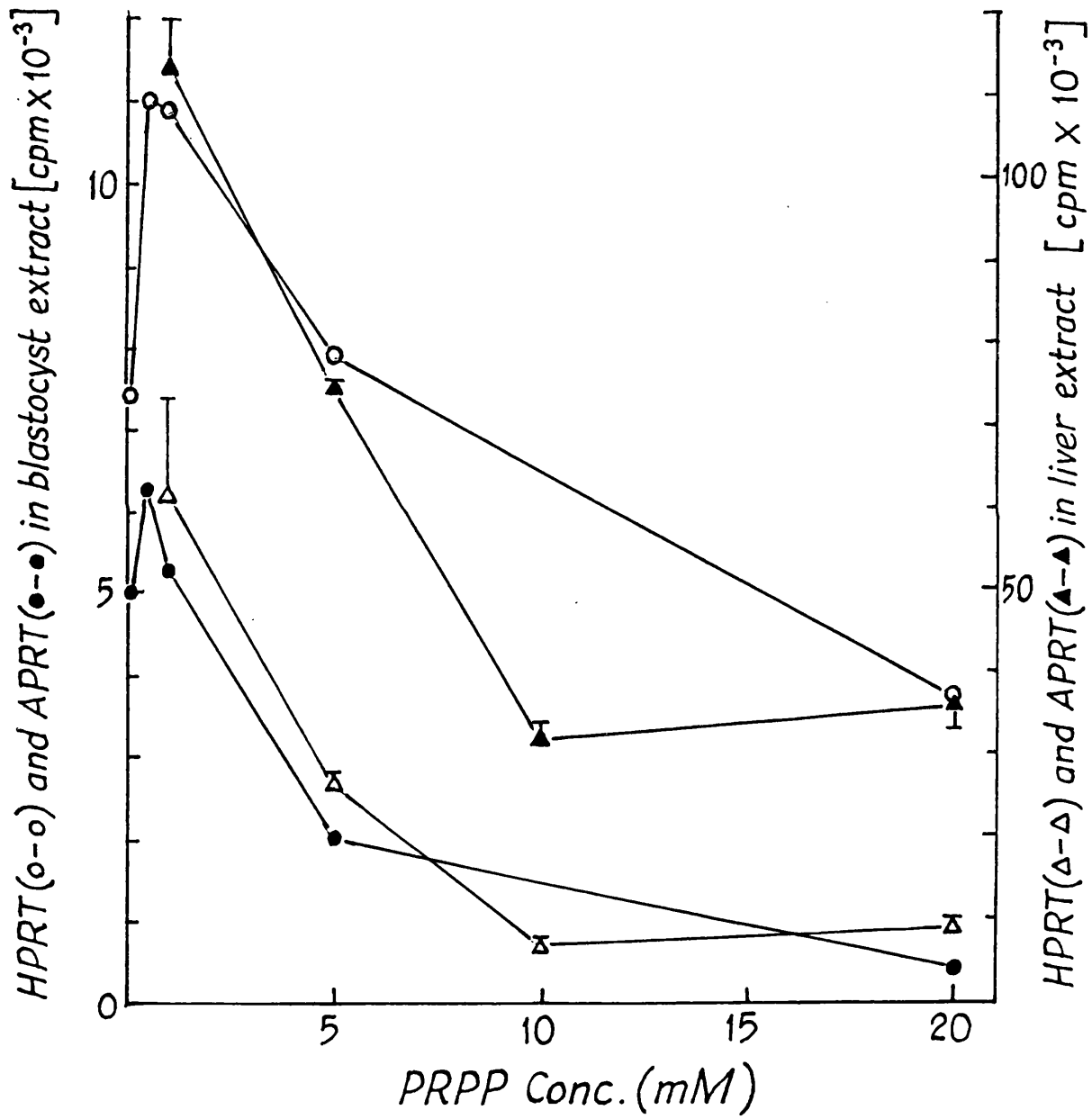
Table 1.2 - Effect of PRPP concentration on HPRT and APRT activities
in liver and blastocyst extracts

| PRPP CON. IN mM/RM | MEAN pM/HOUR ENZYME ACTIVITY IN | | | |
|---|---|---|---|---|
| | LIVER EXTRACT[1] | | BLASTOCYST EXTRACT[2] | |
| | HPRT | APRT | HPRT | APRT |
| 0.1 | - | - | 4.5 | 5.3 |
| 0.5 | - | - | 6.7 | 6.7 |
| 1 | 37.8 ± 6.0 | 122.4 ± 6 | 6.6 | 5.7 |
| 5 | 16.8 ± 0.3 | 80.6 ± 1 | 4.8 | 1.9 |
| 10 | 4.5 ± 0.6 | 31.0 ± 4 | - | - |
| 20 | 5.6 ± 0.4 | 38.2 ± 2 | 2.2 | 0.4 |

[1]Replicate samples were taken for each PRPP concentration to
calculate SE

[2]Only two replicate samples were taken for each PRPP concentration
therefore the SE can not be calculated

inhibit the HPRT and APRT enzyme activities in both liver and blastocyst extracts. However, omission of this substrate results in complete loss of precipitable counts (data not shown). The sensitivity of the reactions to PRPP concentration could explain some of the day to day variability observed in actual enzyme activities. Since PRPP must be fresh and since it is expensive, some variability from one experiment to another is inevitable when weighing milligram amounts. However, all comparisions are made within the experiment and each experiment is internally consistant. Therefore the variability does not affect the conclusions drawn. A major advantage of the assay is that it can be used as a double microassay which simultaneously measures the activities of both HPRT and APRT in a single reaction mixture. A comparison of HPRT:APRT ratios eliminates sampling error and allows comparison between samples.

The assay has been used throughout the work presented in this thesis and the same procedure has been followed as described in this chapter except that the incubation time and specific activities may vary from experiment to experiment (these variations will be noted in each experiment).

## 1.3.2 Profile of HPRT activity and effect of $\alpha$-amanitin in:
## (i) fertilized embryos

The increase in HPRT activity due to translation of stored maternal mRNA and the onset of transcription and translation of the embryonic HPRT gene in fertilized preimplantation mouse embryos have been reported earlier by Harper & Monk (1983). Similar experiments

**Figure 1.4**  Effect of $\alpha$-amanitin treatment on HPRT activity during

preimplantation development of fertilized embryos

1-cell fertilized eggs were cultured in  medium 16 (———O)

Samples were transferred to 10 µg/ml of $\alpha$-amanitin at

different times (———●)

See text for experimental detail

Figure 1.4
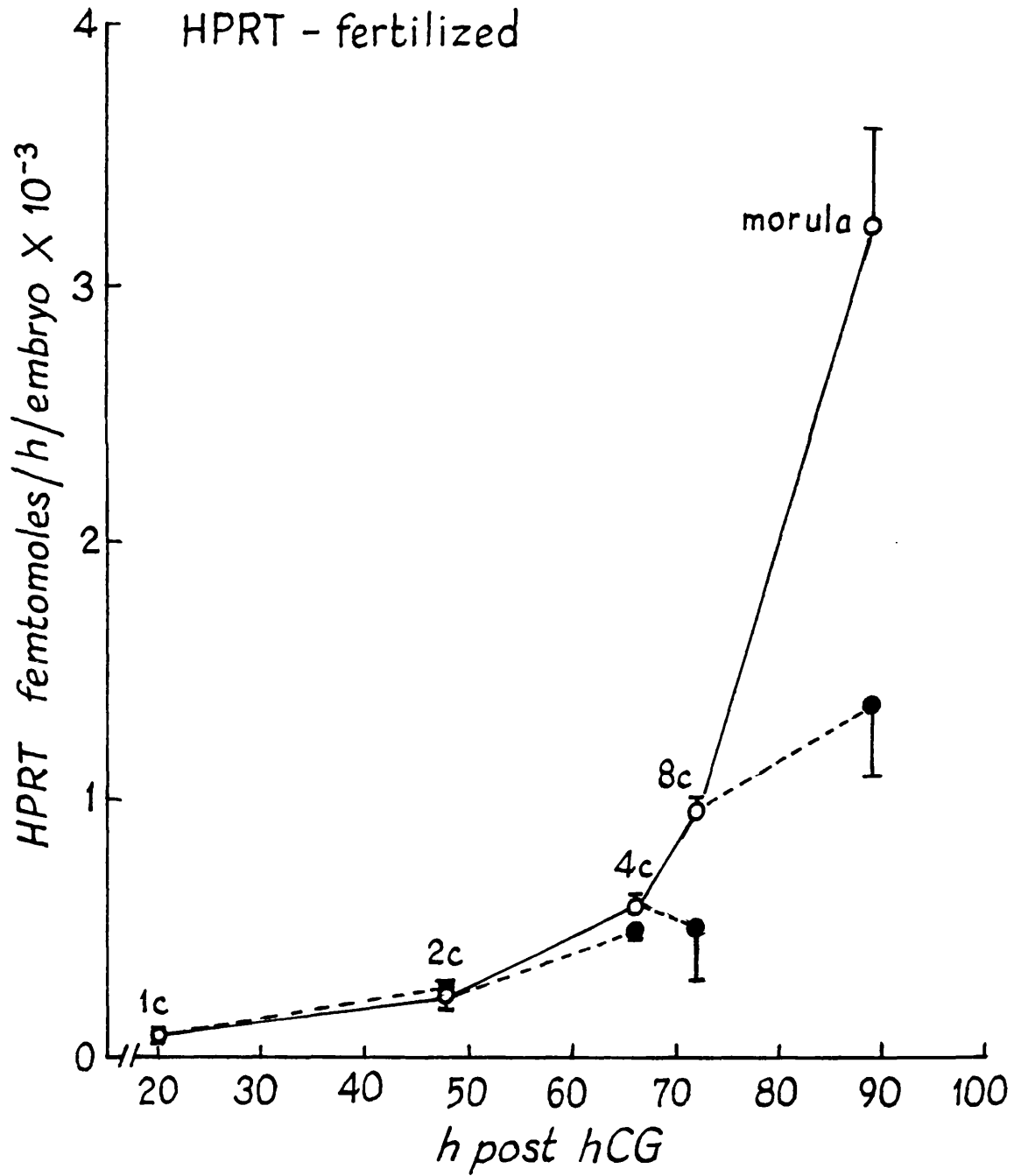
were repeated to confirm and extend the earlier work. Figure 1.4 and table 1.3 show a significant increase in HPRT activity from the 1-cell to the 2-cell stage. Burgoyne, Harper & Monk (unpublished data) showed a similar increase in HPRT activity even in YO embryos, suggesting that the increased enzyme activity is due to the presence of stable maternal mRNA in the egg cytoplasm. To test this, mRNA synthesis was inhibited in the presence of 10 $\mu$g/ml of $\alpha$-amanitin. This treatment did not overtly affect very early development as there was no reduction in the rate of first cleavage. When fertilized eggs (20 h.p.hCG) were cultured for 28 hours in the presence of $\alpha$-amanitin, the HPRT activities were the same as in the control. Thus, the significant rise in HPRT activity from 1-cell to 2-cell stage embryos is insensitive to inhibition of transcription. This result suggests that the early increase in HPRT activity is due to translation of new enzyme on pre-existing maternal message.

In order to test the later stages of development, embryos were cultured in $\alpha$-amanitin at different stages as follows: 2-cell to 4-cell (48-66 h.p.hCG), 4-cell to 8-cell (66-72 h.p.hCG) and 8-cell to morula stage (72-89 h.p.hCG). At these later stages very little effect was seen after 18 hours in the drug (If the embryos were cultured continuously in $\alpha$-amanitin, however, very few embryos divided beyond the 2-cell stage, data not shown). HPRT activities of control and experimental cultures were compared and the results are shown in figure 1.4 and table 1.3.

By 66 h.p.hCG the $\alpha$-amanitin treatment shows a slight but significant inhibition in increase of HPRT activity. At 72 and 89

Table 1.3 - Effect of $\alpha$-amanitin treatment on HPRT and APRT activities during preimplantation development of fertilized embryos

| HOURS POST hCG (STAGE OF DEVELOPMENT)[1] | TREATMENT[2] | HPRT*/[3] | APRT*/[4] |
|---|---|---|---|
| 20 (1-cell) | control | 81 ± 4 | 226 ± 20 |
| 48 (2-cell) | control | 228 ± 9 | 187 ± 37 |
| | $\alpha$-amanitin | 233 ± 60 | 228 ± 74 |
| 66 (4-cell) | control | 564 ± 41 | 474 ± 32 |
| | $\alpha$-amanitin | 476 ± 12 | 440 ± 13 |
| 72 (8-cell) | control | 945 ± 34 | 514 ± 80 |
| | $\alpha$-amanitin | 491 ± 204 | 510 ± 65 |
| 89 (morula) | control | 3212 ± 39 | 1349 ± 117 |
| | $\alpha$-amanitin | 1356 ± 274 | 986 ± 140 |

* Mean enzyme activity (femtomoles/h/embryo) + standard error. The values are plotted in figures 1.4 (HPRT) & 1.7 (APRT).
[1] 1 and 2-cell fertilized eggs were harvested in groups of 3, 4 and 8-cell embryos in groups of 2, and morula stage embryos were harvested singly, at the times indicated above.
[2] control: embryos cultured in medium 16. $\alpha$-amanitin: embryos cultured in 10 µg/ml of $\alpha$-amanitin. See text for experimental detail.
[3] Significance tests, HPRT: 1C<2C, P<.05; 4C+ $\alpha$-amanitin<4C, P<.05; 8C+$\alpha$-amanitin<8C, P<.05; morula+ $\alpha$-amanitin<morula, P<.001.
[4] Significance tests, APRT in control embryos; 2C<4C, P<.001; 4C<morula, P<.001; 8C<morula, P<.001.

h.p.hCG the significant inhibition of HPRT activity in the presence of

α-amanitin is more marked. These results indicate that already at the

4-cell stage some of the increase in HPRT activity is dependent on

newly synthesized embryonic mRNA. From the 4- to 8-cell stage and

from the 8-cell to the morula stage the increase in HPRT activity is

primarily on embryonic mRNA. The residual rise in HPRT activity from

the 8-cell to the morula stage in the presence of α-amanitin suggests

that translation of the enzyme may continue on pre-formed embryonic

mRNA in presence of the drug. A corollory of this is that the

embryonic mRNA must be stable for some period after the addition of

the drug (the intermediate points from 72 hours to 89 hours were not

analysed so the period of stability is not known). These experiments

do not indicate the precise time at which translation of HPRT on

maternal message ceases or the exact period of transition from

maternal to embryonic message. They suggest an earlier onset of

transcription of the embryonic HPRT gene than previously reported

(Harper & Monk, 1983). Some embryonic message for HPRT is synthesized

prior to the 4-cell stage (Paynton et al, 1988) and it is conceivable

that embryonic HPRT mRNA is synthesized as early as the 2-cell stage,

but it is not immediately translated into active enzyme at a

detectable level.


## (ii) parthenogenetic embryos


Diploid parthenogenetic embryos develop slightly more slowly than

control fertilized embryos (further details in chapter 2). During

preimplantation development, the parthenogenotes show a similar

pattern of increased HPRT activity as do the fertilized embryos.

**Figure 1.5** Effect of α-amanitin treatment on HPRT activity during

preimplantation development of parthenogenetic embryos

Embryos at different stages were cultured in either medium

16 (—O) or in 10 μg/ml of α-amanitin (---●)

See text for experimental detail.

Figure 1.5
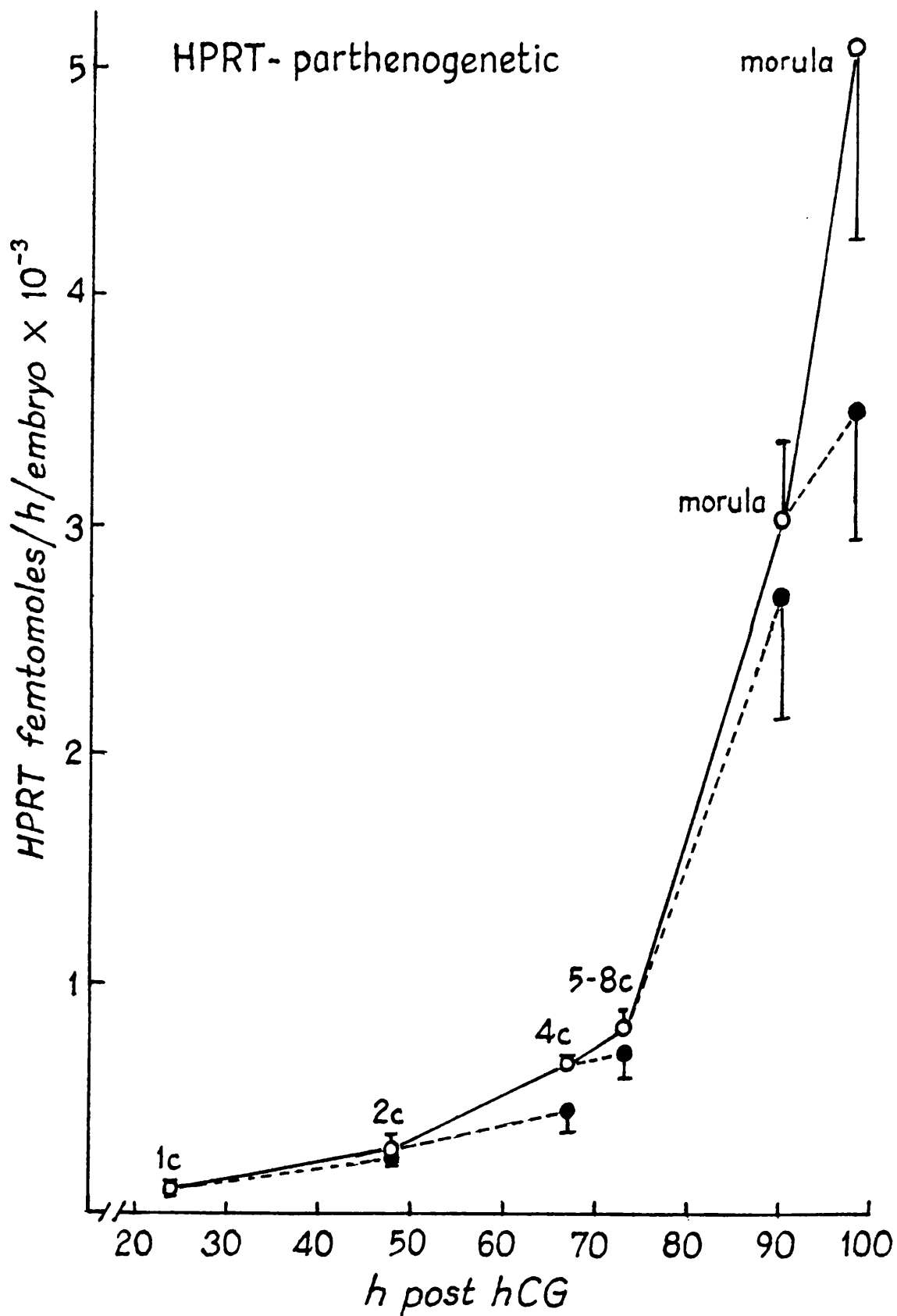
Table 1.4 - Effect of α-amanitin treatment on HPRT and APRT activities
during preimplantation development of parthenogenetic embryos

| HOURS POST hCG (STAGE OF DEVELOPMENT)[1] | TREATMENT[2] | HPRT*/3 | APRT*/4 |
|---|---|---|---|
| 24 (1-cell) | control | 110 ± 10 | 184 ± 11 |
| 48 (2-cell) | control | 268 ± 62 | 119 ± 11 |
| | α -amanitin | 245 ± 31 | 128 ± 19 |
| 67 (4-cell) | control | 664 ± 10 | 275 ± 70 |
| | α -amanitin | 454 ± 81 | 274 ± 45 |
| 73 (5-8-cell) | control | 819 ± 73 | 338 ± 43 |
| | α -amanitin | 708 ± 108 | 225 ± 19 |
| 90 (morula) | control | 3034 ± 332 | 786 ± 98 |
| | α-amanitin | 2709 ± 542 | 745 ± 56 |
| 98 (morula) | control | 5087 ± 842 | 905 ± 149 |
| | α -amanitin | 3498 ± 551 | 591 ± 159 |

*Mean enzyme activity (femtomoles/h/embryo) ± standard error. The values are plotted in figures 1.5 & 1.8.

[1]1-cell parthenogenetically-activated eggs were harvested in groups of 5, 2-cell in groups of 2 and 4-cell, 5-8-cell and morula stage embryos were harvested singly, at the times indicated above.

[2]Control: embryos cultured in medium 16. α-amanitin: embryos cultured in 10 μg/ml of α-amanitin. See text for experimental detail.

[3]Significance tests, HPRT: 1C<2C, P<.01; 2C<4C, P<.05; 5-8C<morula, P<.001.

[4]Significance tests, APRT: 2C<4C, P<.05; 4C<morula, P<.01; 5-8C<morula, P<.001.

Figure 1.5 and table 1.4 show a gradual but significant increase in HPRT activity from the 1-cell to the 4- to 8-cell stage. After 73 h.p.hCG the enzyme activity rises steeply up to late morula stage (98 h.p.hCG). To investigate whether the increase in HPRT activity from 1-cell to 2-cell stage embryos was due to translation of maternal mRNA (as observed in fertilized embryos), the parthenogenotes were cultured in the presence of α-amanitin. The parthenogenetically activated eggs (24 h.p.hCG) cultured for 24 hours in drug show the same rise in HPRT activity as observed in the control. Comparing this result with those in figure 1.4 suggests the translation of inherited maternal mRNA is the same whether onset of development is triggered by fertilization or parthenogenetic activation.

To determine the time of onset of embryonic transcription , the embryos were cultured in α-amanitin from 2-cell to 4-cell (48-67 h.p.hCG), 4-cell to 5- to 8-cell (67-73 h.p.hCG) and 5- to 8-cell to morula stage (73-90) and also the later morula stage (90-98 h.p.hCG). Figure 1.5 and table 1.4 show a gradual increase in HPRT activity in the early cleavage stages of both experimental and control embryos. The increase in HPRT activity becomes sensitive to α-amanitin between the 2-cell and the 4-cell stage (67 h.p.hCG) as observed for the fertilized embryos in figure 1.4. The reason for the greater increase in the presence of α-amanitin from 73 to 90 hours post hCG in this experiment is not known.

## (iii) unfertilized eggs

Unfertilized ovulated eggs were cultured in $\alpha$-amanitin from 22-72 h.p.hCG for different time intervals ranging from 6 to 19 hours. In culture, some of the unfertilized eggs are activated due to temperature decreases and hyaluronidase treatment (Pratt, 1987) and they developed to the 2-cell stage. By 72 hours of culture more than 50% of eggs were degenerated. For the enzyme assay only 1-cell unfertilized intact eggs were harvested.

The results (figure 1.6 and table 1.5) shows a significant increase in HPRT activity from 22 to 41 h.p.hCG in control unfertilized eggs (note that the scale for femtomoles of HPRT of this figure is amplified 10 times in comparison to figures 1.4 and 1.5). The increase in HPRT activity in unfertilized eggs during this period is reproducible and is similar to that observed in fertilized and parthenogenetic embryos from the 1-cell to the 2-cell stage. When cultured in the presence of $\alpha$-amanitin the increase in HPRT activity was not affected suggesting that new enzyme activity is solely due to translation on maternal message. This result provides evidence that the translation of maternal message does not depend on either fertilization or activation. When cultured for longer periods (up to 72 h.p.hCG) no detectable change was observed in HPRT activity (after the initial rise) in both $\alpha$-amanitin-treated and control eggs which shows that the enzyme is quite stable.

**Figure 1.6** Effect of $\alpha$-amanitin treatment on HPRT activity in unfertilized eggs. The unfertilized eggs were cultured in medium 16 (———O) or in 10 µg/ml of $\alpha$-amanitin (---●) See text for experimental detail

Figure 1.6
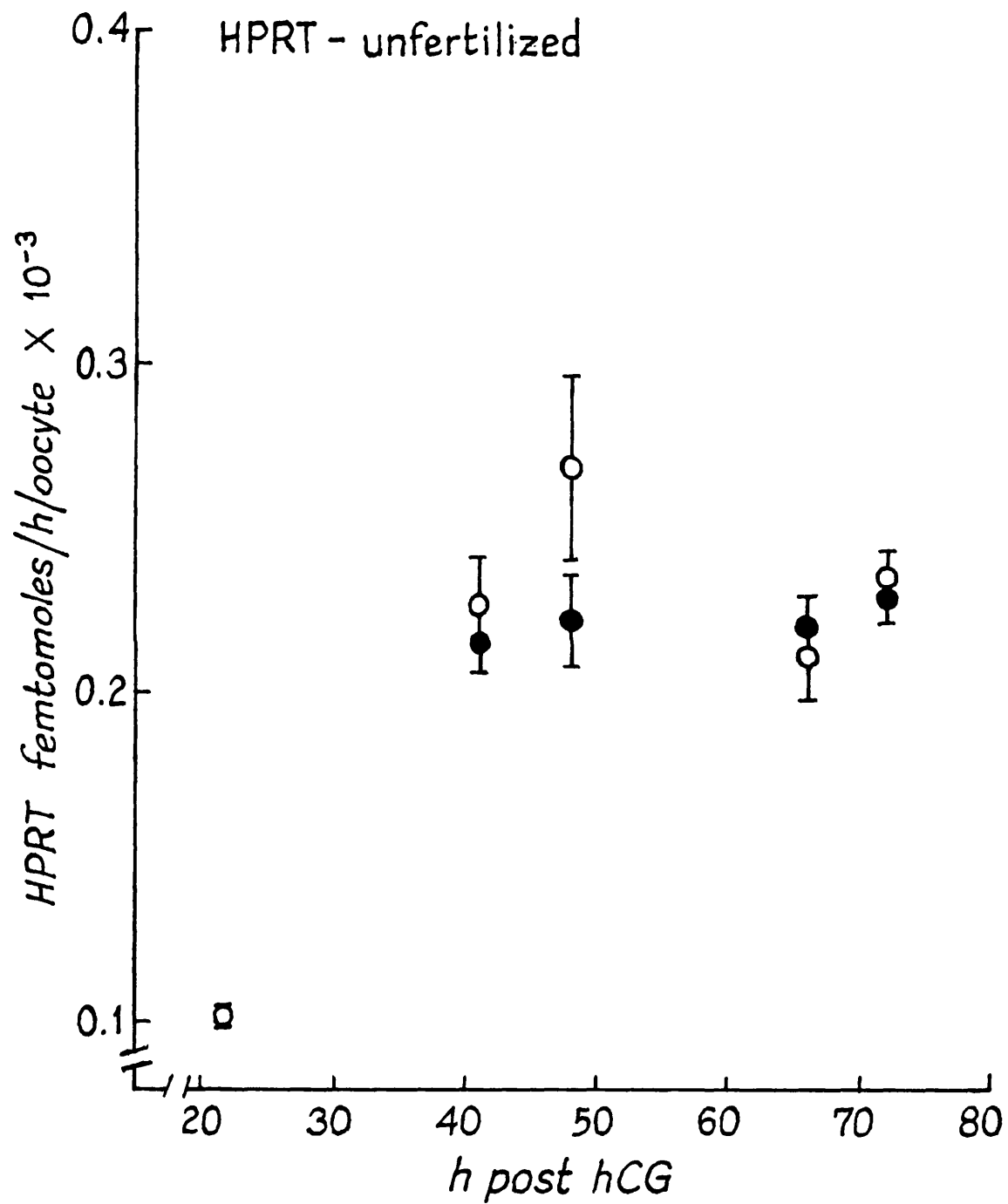
Table 1.5 - Effect of $\alpha$-amanitin treatment on HPRT and APRT
activities in unfertilized eggs

| HOURS POST hCG[1] | TREATMENT | HPRT*/3 | APRT* |
|---|---|---|---|
| 22 | control | 103 ± 2 | 174 ± 10 |
| 41 | control | 226 ± 15 | 126 ± 13 |
| | $\alpha$-amanitin | 215 ± 10 | 161 ± 7 |
| 48 | control | 268 ± 28 | 140 ± 20 |
| | $\alpha$-amanitin | 221 ± 14 | 123 ± 28 |
| 66 | control | 210 ± 13 | 116 ± 9 |
| | $\alpha$-amanitin | 220 ± 10 | 180 ± 3 |
| 72 | control | 234 ± 11 | 125 ± 16 |
| | $\alpha$-amanitin | 229 ± 6 | 153 ± 8 |

*Mean enzyme activity (femtomoles/h/embryo) ± standard error. The values are plotted in figures 1.6 & 1.9.

[1]1-cell unfertilezed eggs were harvested in groups of 2, 3 or 5 at the times indicated above. See text for experimental detail.

[3]Significance tests, HPRT: 22 h.p.hCG<41 h.p.hCG, P<.001.

## 1.3.3 Profile of APRT activity and effect of $\alpha$-amanitin in:

### (i) fertilized embryos

Changes in APRT activity were monitored simultaneously with HPRT activity in fertilized embryos. Figure 1.7 and table 1.3 show the profile of APRT activity in preimplantation development in the presence or absence of $\alpha$-amanitin. Unlike the situation with HPRT, APRT activity does not significantly change from the 1-cell to the 2-cell stage in both experimental and control embryos. From the 2-cell stage, the enzyme activity increases throughout cleavage up to the morula stage. Again, unlike the situation for HPRT, from the 2-cell to the 4-cell stage, and from the 4-cell stage to the 8-cell stage, - amanitin treatment has no effect on the increase in activity. However, from the 8-cell stage to the morula stage the increase of APRT activity is significantly reduced by the presence of $\alpha$-amanitin. Hence, onset of embryonic APRT gene expression only becomes detectable between the 8-cell and the morula stages.

### (ii) parthenogenetic embryos

Similar to fertilized embryos, parthenogenetic embryos show no increase in APRT activity from the 1-cell to the 2-cell stage (figure 1.8). This is in contrast to the increase in HPRT observed in parthenogenetic embryos by the 2-cell stage. From the 2-cell stage, an increase in APRT activity is observed. The increase in APRT activity becomes sensitive to $\alpha$-amanitin treatment from the 4-cell stage, one cleavage cycle earlier than was observed in the fertilized

**Figure 1.7** Effect of $\alpha$-amanitin treatment on APRT activity during

preimplantation development of fertilized embryos

—O = embryos cultured in M16

---● = embryos cultured in 10$\mu$g/ml $\alpha$-amanitin.

See text for experimental detail

Figure 1.7



APRT - fertilized
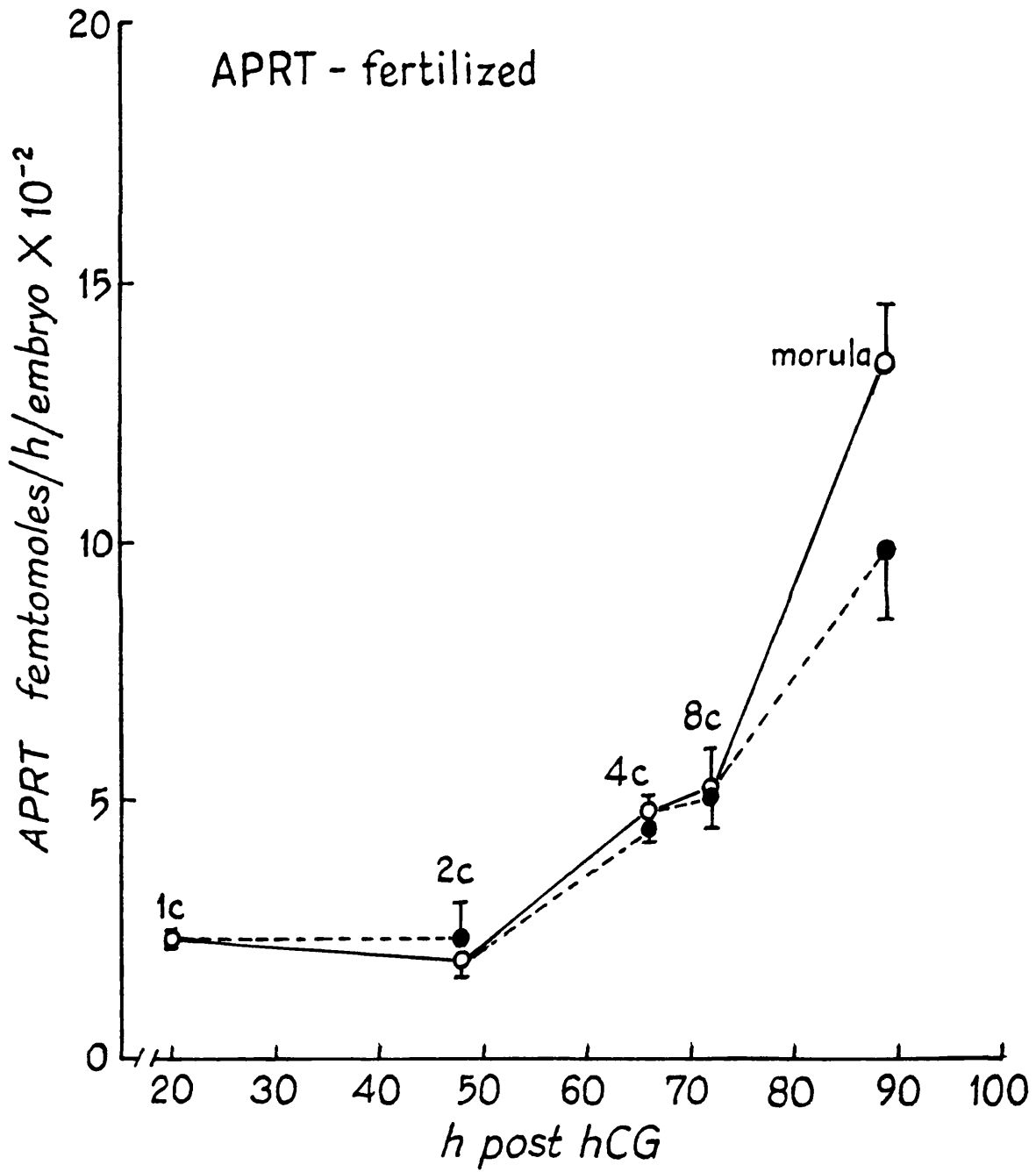
**Figure 1.8** Effect of α-amanitinon treatment on APRT activity in

parthenogenetic embryos

——O = embryos cultured in M16

---● = embryos cultured in 10 μg/ml of α-amanitin

See text for experimental detail

Figure 1.8



Figure 1.8 — APRT parthenogenetic. APRT femtomoles/h/embryo × 10⁻² versus h post hCG. Data points labelled 1c, 2c, 4c, 5-8c, morula, morula.
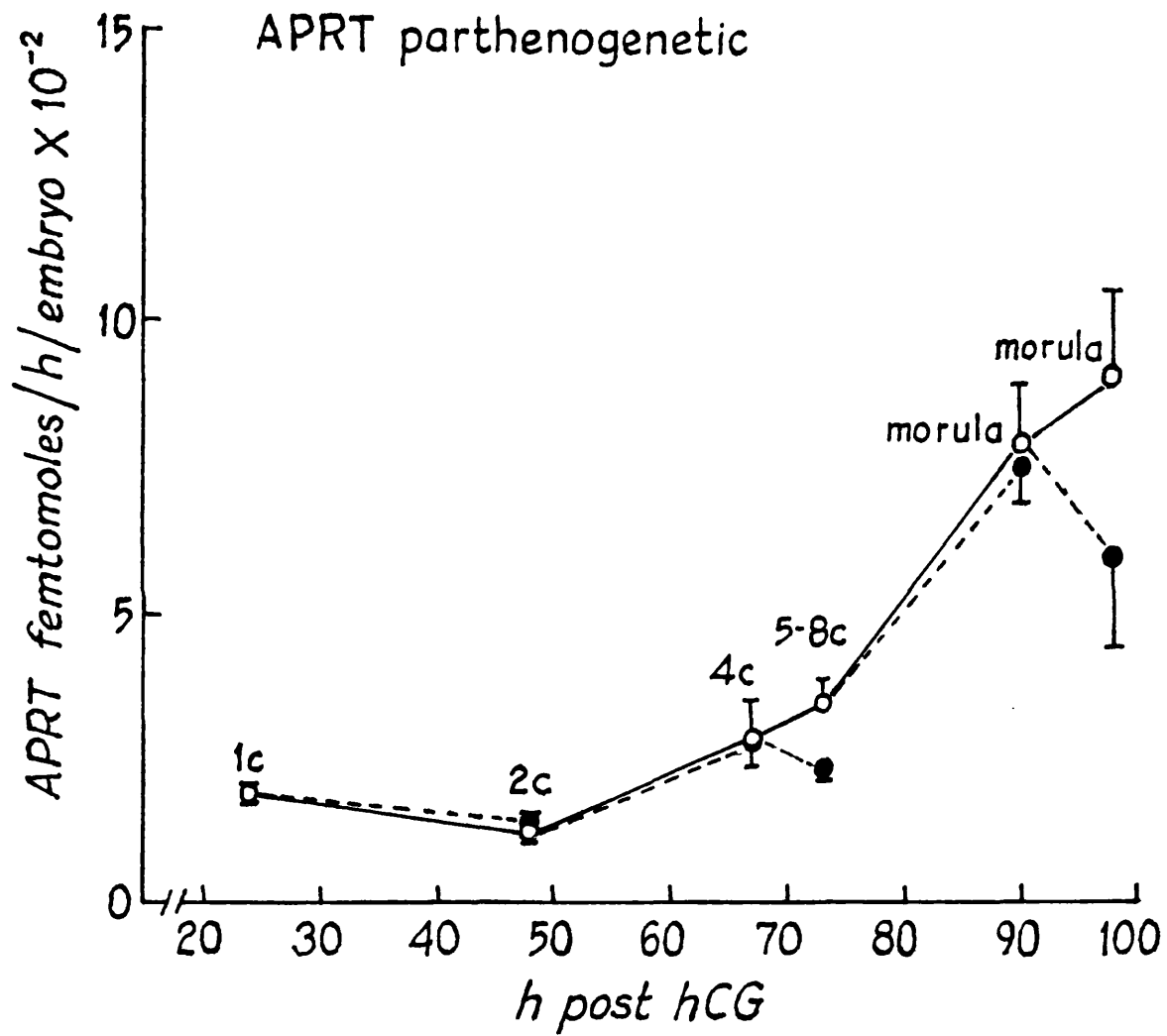
**Figure 1.9**  Effect of α-amanitin treatment on APRT activity in

unfertilized eggs

——O = embryos cultured in M16

---● = embryos cultured in 10 μg/ml of α-amanitin

See text for experimental detail

Figure 1.9
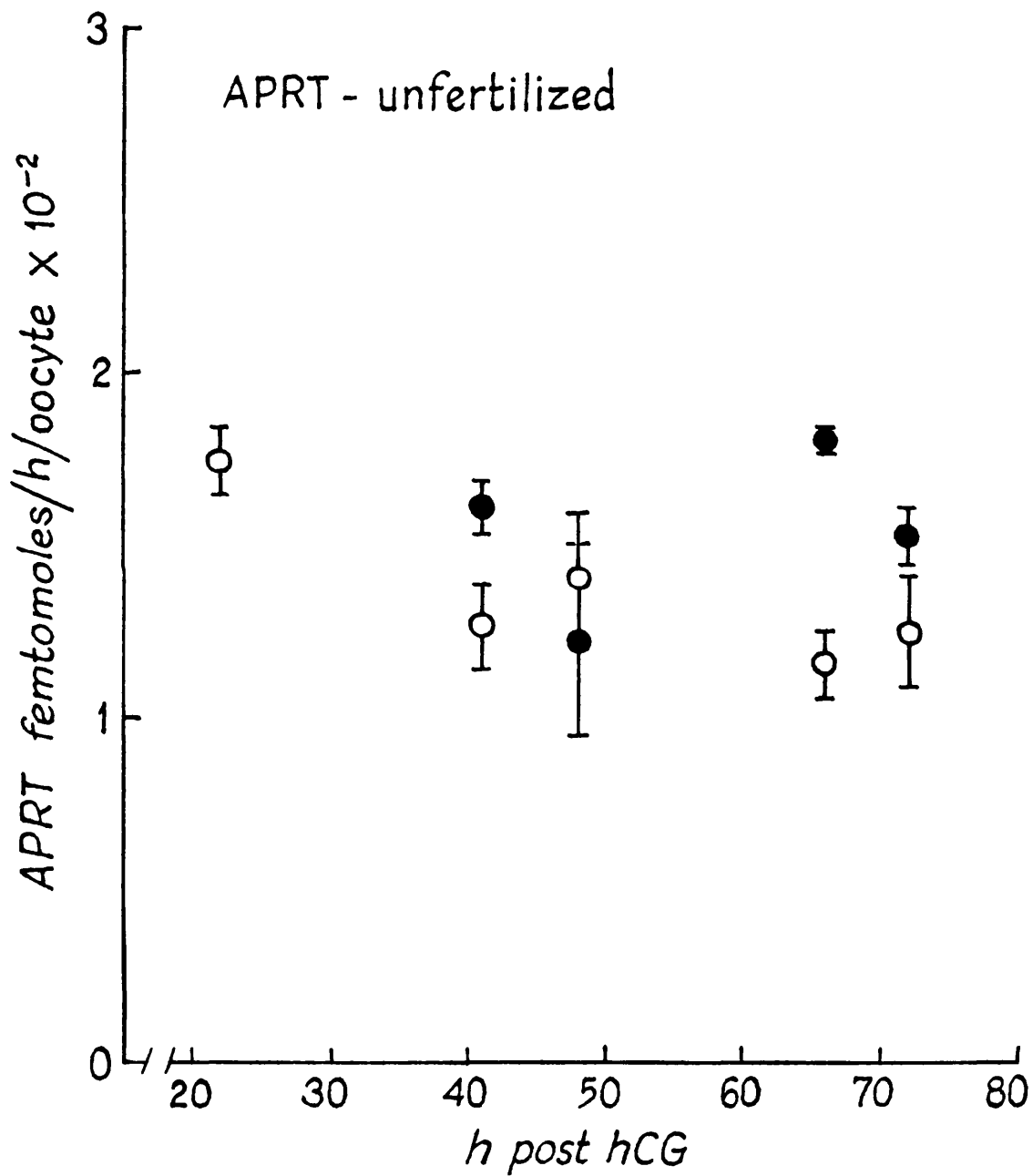
embryos, though still later than the $\alpha$-amanitin sensitivity for the increase in HPRT activity (which is from the 2-cell stage). It appears, therefore, that the embryonic APRT gene is activated as early as the 4-cell stage in parthenogenotes. This is probably also true in fertilized embryos, although significant $\alpha$-amanitin sensitivity was not detected in the experiment shown in figure 1.7, probably due to the variability between replicate samples at this time point

### (iii) unfertilized eggs

Unlike HPRT, there is no increase in APRT activity in unfertilized eggs in culture from 22 to 41 h.p.hCG. The enzyme activity in the egg cytoplasm remains constant without further increase, and without degradation throughout culture of the eggs up to 72 h.p.hCG (figure 1.9, table 1.5).

## 1.4  DISCUSSION

Early mouse development may be jointly regulated by two programmes. The oocyte programme, presumably activated by the LH (lutenising hormome) surge during the first meiotic division and the activation programme, initiated by sperm penetration, or by parthenogenetic activation. This later programme, may include activation of previously silent maternally-inherited information in addition to the activation of the embryonic programme, with the appearance of new embryonic transcripts.

Early studies on macromolecular synthesis relied on the use of inhibitors of DNA, RNA or protein synthesis. Although techniques are now available to directly quantitate specific macromolecules, inhibition studies may be useful where the amount of biological material is limiting, as in preimplantation development. Although there are problems with possible side effects with the use of toxic inhibitors, it has been shown that $\alpha$-amanitin is an active inhibitor of mRNA synthesis during preimplantation development. Levey & Brinster (1978) have shown that 10 $\mu$g/ml of $\alpha$-amanitin inhibits RNA polymerase II activity in the blastocyst within an hour, but no precise information is available for other cleavage stages.

### 1.4.1  Oocyte and activation programmes of HPRT expression

The results presented in this chapter supports the previous report by Harper & Monk (1983) and confirm that the increase in HPRT activity during early cleavage stages is insensitive to $\alpha$-amanitin

treatment and hence is due to translation of maternal mRNA. It appears that $\alpha$-amanitin does enter the early embryonic cells since eggs fertilized in vitro in the presence of $\alpha$-amanitin show inhibition of the synthesis of new polypeptides characteristic of the 2-cell stage (Braude et al, 1979). The role of maternal mRNA in directing increase in HPRT activity is further supported by several other lines of evidence from our laboratory, e.g. XO embryos have equivalent HPRT activity to XX embryos up to the 8-cell stage (Monk & Harper, 1978), YO embryos show an increase in HPRT activity (Harper, Burgoyne & Monk, unpublished).

The fertilized and parthenogenetic embryos in this study showed an increased HPRT activity from the 1-cell to the 2-cell stage. Moreover, similar increase in HPRT activity was observed in unfertilized eggs during this period. In all three cases, this early increase in HPRT activity was insensitive to $\alpha$-amanitin treatment, suggesting that the new enzyme activity is maternally-coded and also that it is synthesized independently of fertilization or activation. Howlett and Bolton (1985) showed increased synthesis of certain polypeptides in mouse oocytes irrespective of fertilization. This result suggest that at least part of the maternal programme in development becomes activated in the growing oocyte and continues after development begins as well as in the aging oocytes.

The onset of embryonic HPRT activity is detectable in both fertilized and parthenogenetic embryos by the 4-cell stage when increase in HPRT activity is seen to be sensitive to $\alpha$-amanitin treatment. This period coincides with the appearance of the embryonic

HPRT mRNA reported by Paynton et al (1988). This result also shows that onset of the embryonic HPRT gene appears at the same developmental stage whether the embryos were fertilized or parthenogenetically activated.

## 1.4.2  Oocyte and activation programmes of APRT expression

The study of APRT activity in preimplantation mouse embryos and aging oocytes showed quite different roles of maternal mRNA and embryonic transcription in early cleavage stages. Firstly, there was no detectable rise in APRT activity in fertilized and parthenogenetic embryos from the 1-cell to the 2-cell stage (figures 1.7 and 1.8). Similarly, no increase in APRT activity was observed in unfertilized eggs when cultured from 22 to 41 h.p.hCG (figure 1.9). This is in contrast to the maternally-derived increase in HPRT activity observed during the same time of development and suggests that these enzymes, though related in function, are independently regulated during the period of oocyte growth.

One possibility is that there is no maternal mRNA for APRT. However, on further examination, the significant rise in APRT activity observed between the 2-cell stage and 4-cell stages in both fertilized and parthenogenetic embryos is insensitive to α-amanitin treatment. This somewhat surprising result suggests that fertilization or activation triggers the translation of masked maternal mRNA which was previously silent in the egg. In support of this interpretation, no such increase is observed in aging oocytes.

The onset of embryo-coded APRT was detected around the 5- to 8-cell in parthenogenetic embryos, earlier than in fertilized embryos (figures 1.7 and 1.8). This was also observed for HPRT. However, the difference in times of onset of the embryonic APRT observed in these two classes of embryos is not thought to be real but due to a greater variability in values for the fertilized samples. The embryonic APRT gene may be activated as early as 4-cell stage in both fertilized eggs and parthenogenotes; by the late morula stage the increase in APRT appears to be entirely embryo-coded.

## 1.4.3 Enzyme stability

The HPRT and APRT activities in fertilized eggs remain stable for up to 3 days of culture. A similar prolonged stability of HPRT activity was observed in unfertilized human eggs (Braude et al, 1989). Monk & Harper (1983) previously suggested from an observed reproducible drop in activity that degradation of maternally-inherited HPRT enzyme activity occurs around the morula stage in fertilized embryos. This may be an incorrect conclusion unless the enzyme activity is more stable in unfertilized eggs because degradation itself is triggered by the parthenogenetic activation or fertilization.

## 1.4.4 General conclusions

From the results presented in this chapter, it appears that onset of embryonic HPRT expression occurs earlier (before the 4-cell stage) than APRT activity (earlier than the 8-cell stage) in both fertilized

and parthenogenetic embryos. However, it should be borne in mind that the initiation of embryonic transcription for these two enzymes must occur some time prior to the period of detectable $\alpha$-amanitin sensitivity of active enzyme. The embryonic mRNA (for HPRT and APRT) will be synthesized earlier than detectable enzyme resulting from this mRNA and there may be a lag between transcription and translation. There is evidence of delays between transcription and translation of messages required at blastulation (Braude, 1979; Schindler & Sherman, 1981). There will also be a time when the embryonic mRNA has been transcribed and translated into active enzyme but the level of activity is too low to be detected above the maternally-inherited enzyme or enzyme synthesized from the maternal mRNA.

In these studies, which were necessary to define the profiles of HPRT and APRT activities prior to experimental manipulation of genes in early embryos, some interesting observations have been made. The timing of onset of embryonic HPRT detected here is earlier than previously reported by Harper & Monk (1983); the timing of onset of embryonic APRT has not been previously reported and it is noteworthy that it is different from that for HPRT. The different modes of regulation of maternal mRNA for HPRT and APRT (activation and fertilization programme) are particularly interesting. In the case of HPRT, the increase in activity already occuring in the oocyte continues after fertilization. In the case of APRT, it would appear that the maternal mRNA is in a masked or silent form in the egg and this is unmasked or activated only following initiation of embryonic development.

# CHAPTER 2

## ONSET OF MATERNAL AND PATERNAL GENES IN DEVELOPMENT

## 2.1    INTRODUCTION

### 2.1.1 X-chromosome activity in early development

The transition from oocyte-coded to embryo-coded HPRT and APRT activities during preimplantation development was described in chapter 1. The onset of embryonic HPRT activity occurs following parthenogenetic activation or fertilization at or before the 4-cell stage, earlier than previously claimed by Harper & Monk (1983). Prior to the 2-cell, the HPRT activity is attributable to the translation of stored maternal mRNA inherited from the oocyte. The activation of the embryonic HPRT gene occurs at the same time in both fertilized and parthenogenetic embryos. In the case of APRT, embryonic activity is detectable at the 8-cell stage. By the morula stage both HPRT and APRT activities are entirely embryo-coded.

At the morula stage, it can be demonstrated that two X chromosomes are active in female (XX) embryos since they have twice the HPRT activity as males (XY) (Monk & Harper, 1978; Kratzer & Gartler, 1978). In the course of female mouse embryo development, X-chromosome inactivation (Lyon, 1961) occurs, first in the extra-embryonic tissues, the trophectoderm and the primary endoderm. In these tissues, the paternally-inherited X chromosome is preferentially inactivated (Takagi & Sasaki, 1975; West et al, 1977). Clearly, the two chromosomes are recognised as different in some way with respect to the inactivation process- a form of chromosome imprinting. The imprinting in the X chromosome is a special case of the more general imprinting of the maternal and paternal genomes which is established

during or before gametogenesis. The differential modification of maternal and paternal alleles results in differential expression and is essential for successful balanced development of the embryonic and extra-embryonic tissues in the conceptus. The role, if any, of the preferential paternal X-chromosome inactivation in extra-embryonic tissues is not known.

## 2.1.2 X-chromosome activity and methylation

The DNA modification which imprints and distinguishes the parental genetic material, including the X chromosomes, in sperm and egg, must stably persist throughout DNA replication and cell division in the developing embryo. A possible molecular mechanism would be differential DNA methylation. The maintenance of inactivity of genes on the inactive X-chromosome in adult somatic tissues appears to involve DNA methylation (reviewed in Monk, 1986). Early studies showed that DNA from the inactive X-chromosome would only transform recipient cells defective for HPRT function if the DNA was isolated from cells treated with the demethylating agent, 5-azacytidine (Liskay & Evans, 1980; Venolia et al, 1982). In addition, differential patterns of DNA methylation between the active and inactive X-chromosome have been directly demonstrated (Wolf et al, 1984; Yen et al, 1984; Toniolo et al, 1984; Lindsay et al, 1985). The methylation status of specific genes on the X chromosome in oocytes is difficult to assess due to the limitation in the amount of biological material available. However, Sanford et al, (1984) have shown that the DNA of oocytes is undermethylated for repetitive sequences, both centromeric and dispersed and, moreover, oocytes are globally undermethylated

(Monk et al, 1987). The sperm DNA is found to be globally methylated relative to oocytes and structural genes for differentiated functions are highly methylated in sperm. On the other hand, centromeric sequences are undermethylated in sperm.

## 2.1.3 Onset of expression of maternal and paternal X chromosomes in development.

It has generally been assumed that once the embryonic genome becomes active, whatever may be the events following fertilization, the origin of chromosomes is irrelevant to their function and they are genetically equivalent. As outlined above, the X chromosomes are not equivalent with respect to X-inactivation in the extra-embryonic tissues, and the oocyte and sperm genomes are not equivalent with respect to methylation. In addition, recent investigations (McGrath & Solter, 1984a; Surani et al, 1984) suggest that the gametes transmit information identifying the paternal and maternal origin of the genes which profoundly affects development of embryonic and extra-embryonic tissues. These experiments assess the role of the parental origin of the genomes in re-constituted eggs, which contain only maternal or paternal genomes, by following the development of various tissues from such manipulated eggs in vivo and in vitro.

One possible effect of methylation differences in sperm and egg genes or in the sperm and egg X chromosomes would be on the timing of onset of genetic expression in early development. The experiments to be described here were designed to determine whether there is a detectable time difference in the onset of activation of the maternal

and paternal genomes by assaying the onset of embryo-coded HPRT

activity in embryos of different maternal and paternal X chromosome

constitution.


Initially, three approaches were taken into consideration.


**Approach one:**


Haploid gynogenetic or androgenetic embryos can be obtained by

removing the female or male pronucleus of a fertilized egg. These

embryos will have either maternal or paternal genomes, thus expressing

only the maternally- or paternally-inherited X-linked enzyme HPRT and

the autosomal enzyme APRT. As shown in the diagram (figure 2.1) the

two haploid sets of embryos will have either the maternal X (Xm,

haploid gynogenetic embryos) or the paternal X (Xp, haploid

androgenetic embryos) and either the maternal or paternal set of

autosomes. Yp embryos do not survive. By assaying the onset of

embryo-coded HPRT and APRT activities in these two sets of embryos, it

would appear possible to determine whether the maternal genome is

expressed before the paternal genome or vice versa.


However, the haploid androgenetic embryos do not develop beyond

the 4-cell stage whereas some of the haploid gynogenetic embryos

develop normally during preimplantation development (Surani et al,

1986). Also, the haploid gynogenetic embryos develop at a faster rate

than the haploid androgenetic embryos.

Figure 2.1  Haploid gynogenetic and androgenetic embryos produced from fertilized eggs
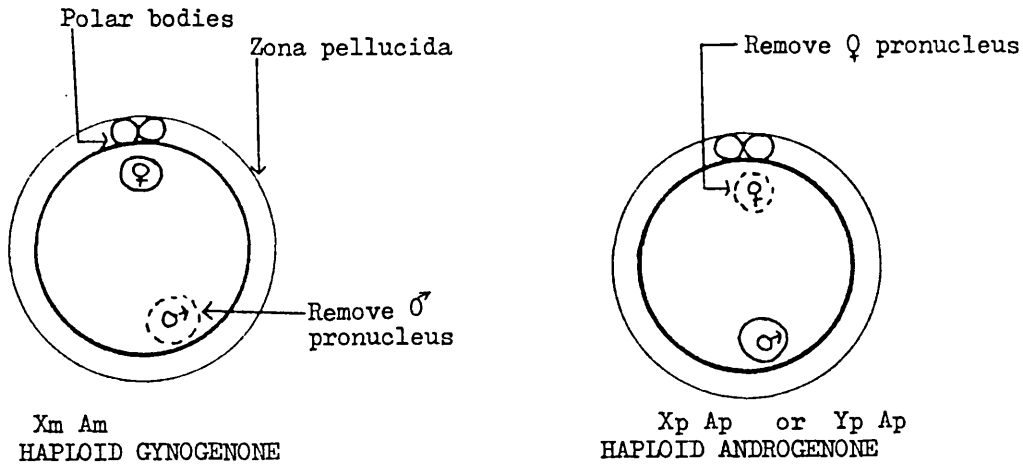


Figure 2.2  Diploid heterozygous gynogenetic and androgenetic embryos produced from fertilized eggs
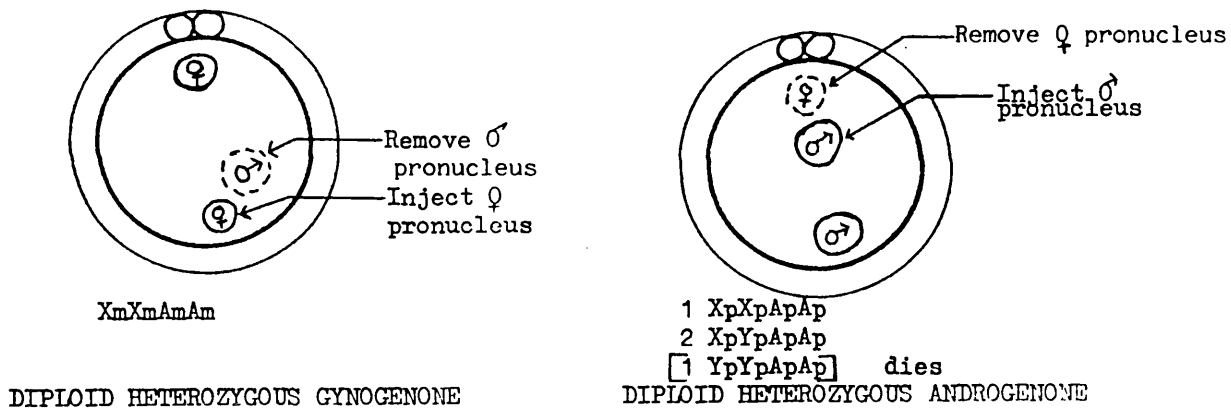


Figure 2.3  Diploid parthenogenotes compared with fertilized eggs
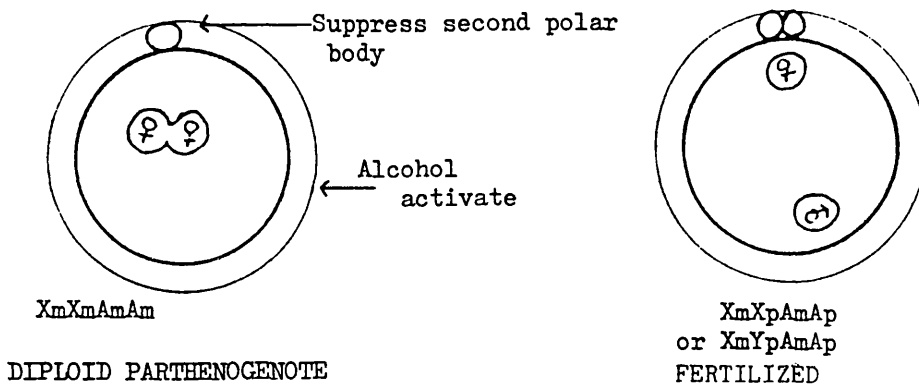
Therefore, for the present project, even if the maternal genome was expressed before the paternal genome, the results would be difficult to interpret between these two sets of embryos due to unequivalent development.

**Approach two:**

Heterozygous gynogenetic and androgenetic embryos can be obtained by removing the male or female pronucleus of a fertilized egg and introducing a female or male pronucleus from another (donor) egg (Surani et al, 1984; McGrath & Solter, 1984a). These two sets of embryos will have either only maternally- or paternally-inherited HPRT and APRT enzymes as shown in figure 2.2. The gynogenetic embryos will be XmXmAmAm and the androgenetic embryos will be in the ratio of 1 XpXpApAp: 2 XpYpApAp: 1 YpYpApAp. Average values for HPRT activity from the two sets of embryos require correction for the number of X chromosomes (multiply the value for androgenetic embryos by 3 and gynogenetic embryos by 2). The APRT values require no correction for the number of autosomes. In this way, it might be possible to compare the enzyme activities at equivalent stages of development for these two sets of embryos to see whether the maternal genome is expressed before the paternal genome or vice versa.

However, the gynogenetic embryos develop better than the androgenetic embryos during preimplantation development. Surani et al (1986) observed that development of biparental gynogenetic embryos to the blastocyst stage was 60%, and for the biparental androgenetic embryos, only 20%.

Therefore, due to unequal development, it would be again difficult to interpret the parental gene expression observed in these two sets of embryos.

**Approach 3:**

In the mouse, embryos with maternal genomes alone can be derived by artificial activation <u>in vitro</u> (by short incubation in 7% ethanol). These haploid embryos begin preimplantation development. In the presence of cytochalasin B following activation, the extrusion of the second polar body may be suppressed giving rise to diploid parthenogenetic eggs (Niemierko, 1975; Kaufman <u>et al</u>, 1977; Borsuk, 1982). Haploid or diploid parthenogenetic embryos can be compared with normal fertilized ones to compare the onset of expression of maternal and paternal HPRT and APRT activities. As shown in the figure 2.3, the diploid parthenogenetic embryos will be XmXmAmAm (haploid embryos, XmAm) and fertilized embryos will be XmXpAmAp or XmYpAmAp. The corrected values of HPRT activities for the number of X chromosomes can be obtained for the diploid parthenogenetic and the fertilized embryos by multiplying the average HPRT values by 3 and 4 respectively. APRT activities need no correction for comparison. In haploids, the corrected values for HPRT and APRT activities can be obtained by multiplying the average HPRT and APRT values by 6 and 2 respectively.

From the three approaches mentioned above, the third one was considered to be technically feasible. This approach was tried in an

attempt to determine whether there is a difference in time of onset of
maternal and paternal genomes.

## 2.2    MATERIALS AND METHODS

### 2.2.1 Embryo isolation and collection

Eggs were obtained from 4-5 weeks old F1 (CBA X C57BL) female

mice as described in chapter 1.2.1. The mice were superovulated by

intraperitoneal injection of 5 i.u. of pregnant mare serum (Intervet)

and 48 hours later, 5 i.u. of human chorionic gonadotrophin (hCG,

Intervet). Half of them were immediately caged with F1 males to

produce fertilized eggs. The rest of them were kept separately to

obtain parthenogenetic embryos. Fertilized 1-cell eggs were collected

from the oviducts 18-20 hours post hCG injection and cumulus cells

removed by treatment for 5 minutes with hyaluronidase (300 i.u./ml).

The eggs were washed and cultured in droplets of M16 medium

(Whittingham, 1971) under paraffin oil at 37°C in an atmosphere of 5%

$CO_2$-in-air.

Eggs from unmated females were isolated in the same way and

activated parthenogenetically by brief exposure (7 minutes) to 7%

ethanol in M16 medium at 37°C. After two washes in M16 medium, half

of the embryos were transferred to droplets of M16 medium under

paraffin oil in plastic culture dishes (previously equilibrated in a

humidified 5% $CO_2$-in-air atmosphere) and incubated at 37°C to obtain

haploid parthenogenetic embryos. Haploid parthenogenotes were

identified by the presence of an extruded second polar body. The rest

of the ethanol-activated eggs were transferred to 25 μg/ml of

cytochalasin B (Sigma) in M16 medium for 4 hours at 37°C to produce

diploid parthenogenetic embryos as described in chapter 1.2.2.

For each treatment, embryos from a number of litters were usually pooled and then added to culture droplets, via a wash droplet, in groups of 20-30. Embryos were either harvested singly or in groups, in 5 µl PB1/PVP in a 10 µl Drummond microcaps and the ends sealed by melting the glass in a low flame. Samples were stored at -70°C. Supernatant extracts were prepared by freeze-thawing three times followed by centrifugation for 10 minutes at 1500 rpm.

## 2.2.2 Enzyme assay

HPRT and APRT activities were measured in embryo extracts as described in chapter 1.2.6. The specific activity of $^3$H-hypoxanthine was 620 Ci/M and of $^{14}$C-adenine, either 310 or 235 Ci/M. The reactions were incubated for 3 hours at 37°C.

## 2.3    RESULTS

Table 2.1 shows the development of the different classes of embryos cultured in vitro. This assessment is biased towards delayed development at later stages since samples of the better developed embryos are being removed for assay each day. However, the bias applies to all three classes of embryos and some conclusions can be made concerning relative development. The diploid parthenogenetic embryos treated with cytochalasin B in addition to alcohol showed better development compared to haploid parthenogenotes. The haploid parthenogenotes did not divide properly after the 4-cell stage, few reached the 8-cell stage, and they failed to form morulae. The diploid parthenogenotes developed nearly as well as fertilized embryos but more detailed observation on the third day of culture showed the diploid parthenogenotes to be somewhat delayed (table 2.1). Due to degeneration of haploid embryos, fewer embryos were available for assay in this class compared to the diploid and fertilized classes.

The number of samples assayed for HPRT in the first preliminary experiment was small. However, the data (table 2.2) shows that the haploid are  delayed in HPRT and APRT expression. In this experiment the diploid parthenogenotes show advanced HPRT expression (2.24 pM/h/embryo) at the morula stage compared to haploid and fertilized embryos which showed 0.52 and 1.37 pM/h/embryo respectively. The relative expression of HPRT (i.e., corrected for the number of X chromosomes per embryo) is 6.70 in diploid, 3.15 in haploid and 5.46 in fertilized. The autosomal enzyme APRT also showed comparatively higher activity in the diploid parthenogenotes (0.17 pM/h/embryo) than

Table 2.1 – Development of diploid and haploid parthenogenotes and fertilized embryos in culture[a]

| EMBRYO CLASS | Day 1 1C | Day 2 deg | Day 2 1C | Day 2 2C | Day 3 deg | Day 3 1-2C | Day 3 3-4C | Day 3 5-8C | Day 3 9-16C | Day 4 deg | Day 4 1-2C | Day 4 5-8C | Day 4 mor | Day 5 deg | Day 5 2C | Day 5 bla |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DIPLOID No | 41 | 18 | – | 23 | 18 | – | 9 | 8 | – | 18 | – | – | 11 | 18 | – | 8 |
| HAPLOID No | 41 | 17 | 2 | 22 | 17 | 7 | 13 | – | – | 17 | 7 | 4 | – | 19 | 3 | – |
| FERTILIZED No | 37 | 7 | – | 30 | 7 | – | 2 | 22 | – | 10 | – | – | 15 | 10 | – | 9 |

*Column group header: DAYS OF CULTURE, stage[b] (Days 1, 2, 3, 4, 5)*

[a] The number of embryos scored is decreasing because some are being harvested for assay each day

[b] deg = degenerating; mor = morula; bla = blastocyst harvested for assay each day

Table 2.2 – HPRT and APRT activities in diploid and haploid parthenogenotes and in fertilized embryos

| Embryo class | Days of culture (h.p.hCG) | Development stage | HPRT pM/h (no.emb/sample) | Relative HPRT expression corrected for no.of X chromosome[1] | APRT pM/h /embryo | Relative APRT corrected for no.of autosome sets |
|---|---|---|---|---|---|---|
| Diploid | 2(48) | 2C | 0.05 (6) | assume maternal not corrected | 0.02 | — |
| Haploid | | 2C | 0.07 (6) | | 0.02 | |
| Fertilized | | 2C | 0.11 (6) | | 0.03 | |
| Diploid | 3(72) | 4C | 0.34 (6) | 1.01 | 0.05 | — |
| Haploid | | 4C | 0.17 (6) | 1.04 | 0.04 | 0.07 |
| Fertilized | | 9-16C | 0.36 (6) | 1.45 | 0.05 | — |
| Diploid | 4(97) | mor | 2.24 (6) | 6.70 | 0.17 | — |
| Haploid | | 2.2C+4.4-8C | 0.52 (6) | 3.15 | 0.05 | 0.09 |
| Fertized | | mor | 1.37 (6) | 5.46 | 0.12 | — |
| Diploid | 5(122) | bla. | 2.15 (2) | 6.45 | 0.13 | — |
| Haploid | | 2C | 0.06 (6) | .37 | 0.02 | 0.04 |
| Fertilized | | bla. | 1.30 (9) | 5.21 | 0.14 | — |

Average HPRT values per embryo were corrected to give comparitive HPRT expression per X chromosome
[1]Diploid parthenogenotes XmXnAmAn, 4 X chromosomes (all maternal) per 2 embryos,
multiply x 3

Haploid parthenogenotes XmAn    , 2 X chromosomes (all maternal) per 2 embryos,
multiply x 6

Fertilized XmXpAmAp and XmYpAmAp, 3 X chromosomes (2 maternal, 1 paternal) per 2 embryos,
multiply x 4

Table 2.3 - HPRT and APRT activities in diploid and fertilized embryos

| Embryo class | Development stage[1] (no.emb./sample) | Days of culture (h.p.hCG) | Av.pM/h/emb. for HPRT (no. samples) | [2]Relative HPRT expression ± SE | Av.pM/h/emb. APRT ± SE | [3]HPRT/APRT ±SE |
|---|---|---|---|---|---|---|
| Diploid | 4C (5) | 3 (72) | 0.14 (3) | 0.42 ± .04 | .03 ± .003 | 14.5 + 1.6 |
| | 9-16C (1) | 4 (96) | 1.14 (5) | 3.43 ± .58 | .14 ± .015 | 24.4 + 3.3 |
| | mor (1) | | 1.36 (5) | 4.10 ± .37 | .17 ± .012 | 24.8 + 1.7 |
| | bla (1) | 5 (120) | 1.02 (4) | 3.05 ± .31 | .13 ± .002 | 28.1 + 2.1 |
| | exp bla (1) | | 1.52 (4) | 4.55 ± .22 | .17 ± .019 | 22.9 + 2.9 |
| Ferti-lized | 4-8C (5) | 3 (72) | 0.14 (3) | 0.57 ± .05 | .06 ± .006 | 9.2 + 1.3 |
| | mor (1) | 4 (96) | 1.18 (3) | 4.73 ± 1.2 | .22 ± .047 | 25.8 + 2.4 |
| | bla (1) | | 0.94 (10) | 3.76 ± .36 | .15 ± .005 | 21.6 + 2.8 |
| | exp bla (1) | 5 (118) | 1.11 (7) | 4.42 ± .87 | .19 ± .003 | 22.8 + 1.4 |

[1]mor=morula; bla=blastocyst; exp bla=expanded blastocyst
[2]Relative HPRT expression corrected for number of X chromosomes
[3]Relative HPRT/actual APRT

in the haploid (0.05 pM/h/embryo) and fertilized (0.12 pM/h/embryo) embryos, on the fourth day of culture. With these encouraging results, future experiments were designed with more embryo samples to measure HPRT activity more accurately and to see if the difference is significant. Since there is a rapid increase in HPRT activity on the fourth day of development in vitro (see chapter 1.3.2 and figure 1.4 the accurate timing and staging of embryos is important in comparisons among the different classes of embryos. In the first experiment the embryos were compared at equivalent times post hCG.

A similar experiment was repeated with an increased number of diploid parthenogenotes and fertilized embryos. Haploid embryos were omitted from the experiment as they do not develop well. The result (table 2.3) shows that, although the diploid parthenogenetic embryos were slower in development, the uncorrected HPRT/embryo value was slightly higher at the morula stage compared to the fertilized embryos at an equivalent time of culture after hCG. When corrected for equivalent numbers of X chromosomes, the two sets of embryos showed similar amounts of HPRT activity. Bearing in mind that the fertilized embryos are slightly advanced in development, this result again is consistent with advanced onset of expression of the maternally-derived HPRT gene. In this experiment, diploid and fertilized embryos were harvested at approximately the same developmental stage, the morula, but the exact numbers of cells were not monitored and compared over the relevant period, the fourth day of culture.

In the next experiment embryos were assessed and sampled only on day 4 of culture. Attention was payed to harvest embryos at an

Table 2.4 - HPRT and APRT activities in diploid and fertilized embryos on the fourth day of culture

| Embryo class | Development stage | h.p.hCG | Av.pM/h/emb. for HPRT (no. samples[1]) | [2]Relative HPRT expression ±SE | Av.pM/h/emb. APRT ±SE | [3]HPRT/APRT ±SE |
|---|---|---|---|---|---|---|
| Diploid | 9-16C | 87 | 1.12 (9) | 3.34 + .5 | 1.49 + .2 | 2.21 + .3 |
| | 9-16C | 90 | 0.93 (14) | 2.80 + .2 | 1.49 + .2 | 2.14 + .4 |
| | 17-32C | 93 | 1.58 (14) | 4.73 + .3 | 1.84 + .3 | 2.80 + .2 |
| Ferti-lized | 17-32C | 87 | 1.06 (10) | 4.23 + .2 | 1.77 + .2 | 2.55 + .5 |
| | early.bla | 90 | 1.18 (10) | 4.71 + .3 | 1.35 + .2 | 3.80 + .6 |

[1]One embryo per sample
[2]Relative HPRT expression corrected for number of X chromosomes
[3]Relative HPRT/actual APRT

equivalent stage of development, the 32 cell morula (time post hCG, diploid parthenogenotes 93h, fertilized embryos 87h). The results, in table 2.4, show no significant difference between these two classes of embryos at this stage. Since embryo-coded HPRT expression begins around the 4-cell stage, it is possible that the 32-cell morula stage is too late to see a difference due to possible earlier onset of the HPRT allele on the maternal X (Xm). A careful comparison of earlier stages between these two classes of embryos would be required to draw any definite conclusion. In one experiment (data not shown) 9-to 16-cell in vivo control embryos were compared with in vitro diploid parthenogenotes at the same stage. The parthenogenetic embryos in vitro were around 17 additional hours post hCG compared with the in vivo control embryos. In this experiment the delayed diploid parthenogenotes (with only maternal X chromosomes) again showed higher HPRT activities (at 92 hours post hCG in vitro) compared to fertilized (with maternal and paternal X chromosomes) embryos (75 hours post hCG in vivo). Although this result supports the earlier onset of the HPRT allele on the maternal X chromosome, (Xm), it was difficult to be sure that equivalent stages were being compared and these experiments were not continued.

## 2.4    DISCUSSION

Recent investigations suggest that gametes may differ in more ways than their striking morphological differences. The egg and sperm have different roles to play in early development and both sets of information are required for successful development (Barton et al, 1984; McGrath & Solter, 1984a; Surani et al, 1984). Hence, diploid parthenogenotes with two maternal genomes or two paternal genomes do not survive (McGrath & Solter, 1984a). The maternal genome appears to be more important for development of the fetal components of the conceptus and paternal genome for the development of extra-embryonic tissues. Embryos possessing two paternal genomes may result in hydatidiform moles (Kajii & Ohama, 1977). Another observation highlighting the different roles of the paternal and maternal genomes in early development is the preferential inactivation of the paternal X chromosome in the extra-embryonic tissue of the mouse conceptus.

The differential roles of paternal and maternal alleles also may be evident in newborn mice. Cattanach and Kirk (1985) showed that differential activity of paternally- and maternally-derived chromosome regions in mice was necessary for normal phenotype in live born animals.

Recently, Reik et al (1987) and Sapienza et al (1987) have provided evidence for a mechanism of imprinting and showed DNA methylation differences between maternally- and paternally- inherited transgenes. Some imprinting mechanism involved with endogenous genes

e.g. the T$^h$P-mutation is lethal only when transmitted via the maternal nucleus (McGrath & Solter, 1984b).

Differential parental allele expression at any developmental stage could result in quantitative effects on expression of the two alleles and/or differential timing of onset of parental gene expression. In this chapter an attempt has been made to investigate possible differences in timing of parental gene expression in mouse embryos.

The results (in all three experiments) showed higher levels of expression of uncorrected HPRT/embryo in diploid parthenogenetic embryos. When the values are corrected to equivalent numbers of X chromosomes compared to fertilized embryos, expression of the maternally-inherited gene was higher or equivalent to paternal plus maternal gene expression during cleavage. The tables (2.1, 2.2 and 2.3) show that though the diploids were slower in development, the HPRT activity was either higher (table 2.2) or equal (table 2.3) when assayed after equivalent times of culture and times post hCG. However, to draw a final conclusion, more work would be needed with special attention to staging and timing of embryos. In my experiments, it was difficult to synchronize the embryos due to unequal development and to be sure of the conclusions. Further experiments with carefully staged embryos at earlier times and developmental stages indeed may establish the earlier onset of the maternal allele.

The functional difference of paternal and maternal genomes in development have been reported by several workers but there is little information as to whether there is a difference in timing of onset of gene expression from maternal and paternal genomes during early embryo development. Chapman et al (1971) initially showed that the paternally-derived allele of the gene for enzyme glucose phosphate isomerase (GPI-1) is expressed by the late blastocyst stage. Brinster (1973) showed onset of expression of paternal GPI-1 earlier, by the 8-cell stage. In both sets of experiments the embryos were produced by crossing homozygous Gpi-1$^a$/Gpi-1$^a$ females with homozygous Gpi-1$^b$/Gpi-1$^b$ males and paternal gene expression was monitored by the appearance of the BB homodimer and AB heterodimer of this dimeric enzyme. In these experiments the detection of maternal gene expression was difficult because of the high level of stored maternal enzyme in the oocyte.

The discovery of a third allele Gpi-1$^c$ by Padua et al (1978) provided West and Green (1983) an opportunity to study the activation of maternal and paternal allele in embryos produced by fertilizing heterozygous Gpi-1$^a$/Gpi-1$^b$ females with homozygous Gpi-1$^c$ males. Their results suggest that the paternally-derived Gpi-1 allele is expressed between 2 1/2 and 3 1/2 days post coitum. A Similar experiment was repeated by Gilbert and Solter (1985) in mouse embryos produced by transplanting pronuclei between two strains of mice with different Gpi-1 alleles. Their results suggest that both the maternal and paternal genes are activated simultaneously and detected on day 4 of embryogenesis. Duboule and Burki (1985) also suggest that the maternal allele is expressed at the same time as the paternal allele.

In addition to GPI-1, another X-chromosome-linked gene, PGK-1, has been used to study the relative timing of the onset of maternally- and paternally-derived genes. Krietsch et al (1982) suggest earlier onset of the maternal allele compared with the paternal allele and similar issue has been also been raised by Papaioannou et al (1981).

Unlike GPI-1 and PGK-1, isomeric forms of HPRT were not available to us. In this chapter an attempt was made to study the relative timing of onset of paternal or maternal gene expression by assaying the onset of embryo-coded HPRT activity in embryos of different parental X chromosome constitution. However, due to an increasing interest in the manipulation of gene expression in the preimplantation embryo, it was considered that enough ground work had been done in preparation for the experiments reported in the following chapters, and the question of timing of maternal and paternal gene expression was not continued.

# CHAPTER 3

## EXPRESSION OF INJECTED HPRT MINIGENE IN THE
## PREIMPLANTATION MOUSE EMBRYO

## 3.1 INTRODUCTION

### 3.1.1 Expression of genes injected into fertilized eggs

In the investigation of the early mammalian embryogenesis it is important to understand the role played by a particular gene or genes in the developmental programme. The small amount of biological material of the mammalian egg and preimplantation embryo render molecular studies extremely difficult. Therefore, the study of transcriptional regulation, for example, specific promoter function and promoter induction requires a sensitive assay to detect specific gene activity. One system of gene expression in early development that is amenable to study is the onset of HPRT gene activity. A highly sensitive biochemical double microassay is available that detects the activities of HPRT and APRT in a single egg, preimplantation embryo or even a single blastomere of the cleavage-stage embryo (Monk, 1987; Monk & Handyside, 1988; also see chapter 1).

Plasmids containing eukaryotic genes have been injected into frog or mouse oocytes to study the nucleotide sequences required for synthesis of functional mRNAs (Grosschedl & Birnstiel, 1980; Dierks et al, 1981; Wickens et al, 1980; McKnight et al, 1981; Brinster et al, 1981). Brinster et al (1982) constructed a plasmid with the viral thymidine kinase gene linked to the metallothionein-I promoter and injected it into fertilized mouse eggs for quantitative analysis of transient expression at the 2-cell stage. However, there is no information on the expression of the microinjected plasmid DNAs in the later cleavage stages. Therefore, an experiment was designed to study

the activity of exogenous HPRT genes, injected at the 1-cell stage, throughout preimplantation development of the mouse embryo.

### 3.1.2 Embryo culture and choice of mouse strain

For experiments monitoring gene expression throughout preimplantation development, it was important to use fertilized eggs which developed well in culture to the blastocyst stage. Mammalian embryos, including human embryos, can be cultured _in vitro_ through several cleavage stages and beyond, to the blastocyst stage (Whittingham, 1975 & 1979). In the mouse, complete development through the preimplantation period (1-cell to the blastocyst stage) is possible (Whitten & Biggers, 1968) without affecting subsequent embryonic viability after transfer (Whittingham, 1975; Hoppe & Pitts, 1975). However, development _in vitro_ from the 1-cell to the blastocyst stage is restricted to certain inbred strains and F1 hybrids (Whittingham, 1975; Whitten & Biggers, 1968; Biggers, 1971; Shire & Whitten, 1980). In most randomly-bred strains the 1-cell embryo becomes blocked at the 2-cell stage _in vitro_ and it will only continue development if transferred back to the oviduct (Whittingham & Biggers, 1967). In the experiments reported in this thesis, CBA x C57BL F1 eggs were used for gene injection; in one series of experiments random bred MF1 eggs were also used for comparison.

### 3.1.3 Enzyme profile in preimplantation development

As mentioned in chapter 1, a number of enzymes increase their activity during preimplantation development probably due to early

activation of embryonic gene transcription e.g. malate dehydrogenase, fructose-1,6-diphosphate aldolase (Epstein et al, 1969), aspartate aminotransferase (Moore & Brinster, 1973), $\beta$-glucosidase (Chapman et al, 1977), HPRT and APRT (Harper & Monk, 1983) and adenosine deaminase (Benson & Monk, 1988)

There is another class of enzymes that are inherited at high levels in the egg cytoplasm and are stable for 2.5 days. Thereafter, they show an exponential decline in activity initiated at around the 8-cell stage e.g. lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PD) (Epstein et al, 1969) and phosphoglycerate kinase (Kozak & Quinn, 1975). By immunological identification studies, Spielmann et al (1974) have shown that the lowered activity of both LDH and G6PD are due to the actual loss of enzyme molecules. Epstein et al (1969) have suggested that a specific degradation process may be involved.

Evidence has been presented for the presence of stable maternal mRNA in the egg, encoding for specific proteins (Braude et al, 1979) or enzymes (Harper & Monk, 1983) appearing during early mouse development. However, there appears to be a striking degradation of maternal RNA (40%) detectable on the first day of preimplantation development, the remaining RNA was stable on the second day and then declined by an additional 30% on the third day of development to the blastocyst stage (Bachvarova & De Leon, 1980; see also chapter 1).

In the case of HPRT there is significant loss of maternal message by the late 2-cell stage (Paynton et al, 1988) but the maternally-

inherited enzyme is more stable (chapter 1) and, in the human, is stable right up to the blastocyst stage (Braude et al, 1989). In the mouse, the embryonic HPRT gene is activated prior to the 4-cell stage (chapter 1), and thereafter HPRT activity continues to increase due to synthesis of enzymes on embryo-coded mRNA.

### 3.1.4 Overexpression of injected genes in blocked 2-cell embryos

In the course of experiments following activity of the bacterial enzyme XPRT (xanthine phosphoribosyl transferase) synthesized from cloned XPRT genes injected into mouse eggs, Lovell-Badge (unpublished) observed an enormous increase (to 30 pM/embryo/hour) in XPRT activity after 96 hours of culture in embryos blocked at the 2-cell stage. A similar increase in HPRT activity is shown in the experiments reported in this chapter when, occasionally, the eggs injected with cloned HPRT DNA, remained blocked at the 1- or 2-cell stage. One possibility is that development is blocked and expression is high because these eggs get massive doses of DNA. The other possibility is that degradation processes that might remove the transcripts from the injected DNA do not occur if development is blocked. To settle this question, in experiments reported in this chapter, plasmids were injected into MF1 eggs whose embryos block at the 2-cell stage, and resultant HPRT activity compared after the same periods of culture with that of injected F1 embryos which will develop to the blastocyst stage. This study also examines whether there is an altered profile of endogenous HPRT activity when culture of blocked 2-cell embryos is continued.

### 3.1.5 Promoter function in early mouse embryos

The SV40 large T promoter has been linked to other gene sequences in a number of experiments concerned with the study of gene expression in mammalian cells (Mulligan & Berg, 1980 & 1981; Land et al, 1983) including embryonic stem cells (Lovell-Badge et al, 1985) and production of transgenic mice (Adams et al, 1985). However, several workers say the SV40 promoter does not function efficiently in undifferentiated embryonal carcinoma (EC) cells (Linnenbach et al, 1980; Segal et al, 1979; Swartzendruber & Lehman, 1975).

One of the promoters under study here is the mouse metallothionein-I promoter. There are a number of cases where the metallothionein-I promoter has been fused to other structural genes to study gene expression in mouse eggs (Brinster et al, 1982; Stuart et al, 1984), cell transfection assays (Mayo et al, 1982) and in production of transgenic mice (Palmiter et al, 1982a,b; Palmiter et al, 1983a). The mouse metallothionein-I promoter can be induced by heavy metals and glucocorticoid hormones (Beach & Palmiter, 1981; Hager & Palmiter, 1981; see details in chapter 4). In this chapter, the uninduced metallothionein-I promoter is compared with the SV40 promoter and mouse HPRT promoter. All the three promoter regions were linked to the mouse HPRT structural gene injected into mouse eggs and the promoter function studied by assaying the resulting HPRT gene activity. (For induction of activity in embryos injected with the HPRT minigene driven by the metallothinein promoter see chapter 4).

### 3.1.6 Chromatin structure and expression of injected genes

Several studies suggest that chromatin structure plays a role in eukaryotic gene expression. Earlier work by Weintraub & Groudine (1976) has shown that active gene chromatin is more susceptible to deoxyribonuclease I than the bulk chromatin and specific sites at the 5' and 3' boundaries of active genes are preferentially exposed. These sites of active genes are found to be hypersensitive to deoxyribonuclease I (Wu, 1980; Stalder et al, 1980; Samal et al, 1981; Elgin, 1981), micrococcal nuclease (Samal et al, 1981; McGhee et al, 1981) and specific restriction endonucleases (McGhee et al, 1981; Sweet et al, 1982). When the genes are inactive, the preferential nuclease sensitivity is not observed in those same sequences (Stalder et al, 1980; Wu & Gilbert, 1981). Recent reports show that DNA single-strand-specific reagents, S1 nuclease (Larsen & Weintraub, 1982) and bromoactaldehyde (Kohwi-Shigematsu et al, 1983) selectively react at the nuclease hypersensitive sites of active chromatin and in supercoiled DNA.

Recently several workers have demonstrated that injected supercoiled DNA is a more successful template for transcription than linear DNA in vivo (Harland et al, 1983; Sollner-Webb & McKnight, 1982; Pruitt & Reeder, 1984a,b; Weintraub et al, 1986; Ryoji & Worcel, 1984). To study whether specific DNA topology is required for successful transcription, the thymidine kinase gene in the form of supercoiled plasmid was injected into Xenopus oocytes (Harland et al, 1983). The results showed a high rate of TK gene transcription. However, if the injected supercoiled DNA was linearized in situ by

subsequent injection of restriction enzyme, the synthesis of transcripts was substantially reduced. Similar results were obtained with rDNA and 5SRNA genes in <u>Xenopus</u> oocytes (Sollner-Well & McKnight, 1982; Pruitt & Reeder, 1984a). In addition, certain parameters can be modulated <u>in vivo</u> by the addition of intercalating agents, e.g. ethidium bromide or chloroquin (Pruitt & Reeder, 1984b) or novobiocin (Ryoji & Worcel, 1984). This provides circumstantial evidence that eukaryotic cells may take advantage of the information provided by altered DNA topologies or conformations. In this chapter the expression of active enzyme from supercoiled and linear HPRT minigene constructs is compared in 2-cell embryos.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Plasmid constructions

The construction of the 3 kb minigene (pDWM1) from the 33 kb long

HPRT gene has been described by Melton et al (1986). The minigene

used in these experiments (pDWM1△-638) was derived from the basic

construct by deleting sequences between the Bam HI and Bgl II

restriction sites (figure 3.1A). The expression vector, pMT142

(figure 3.1B), was kindly provided by R.Palmiter. In this vector, the

0.77 kb Eco RI/Bgl II fragment, containing the mouse metallothionein

promoter and the 0.65 kb fragment from the 3'-end of the human growth

hormone gene, containing a polyadenylation signal, were cloned

together in a pBR322 derivative. Plasmid pMT-HPRT (figure 3.1C) was

constructed using a promoterless HPRT minigene obtained by deleting

all 5' flanking sequences up to +63. A Bam HI linker molecule was

then affixed to the 5' end and the promoterless minigene cloned, as a

Bam HI/Eco RI fragment, together with the mouse metallothionein Eco

RI/Bgl II promoter fragment (from pMT142) in plasmid pUC8. Plasmid

pSV-HPRT (figure 3.1D) was constructed by removing the 2.75 kb Bgl

II/Eco RI fragment, containing the neo gene and SV40 RNA-processing

signals, from plasmid pSV2 neo (Southern & Berg, 1982), and inserting

the same promoterless HPRT minigene fragment used in figure 3.1C, thus

bringing the HPRT minigene under the control of the SV40 promoter

fragment. The DNA constructs were prepared by D.Melton.

**Figure 3.1** Construction of plasmid vectors containing the HPRT

minigene   linked to different promoters.

**(A)** pDWM1, the basic mouse HPRT minigene.  Open boxes, HPRT coding

sequence; closed boxes, untranslated regions; thick line, flanking and

intervening sequences; thin lines, vector (PUC8) sequence.  The main

transcriptional start site (+ 1) is indicated.

**(B)** pMT142, the growth hormone gene linked to metallothionein-I

promoter.  Shaded box, mouse metallothionein promoter; stippled box,

the 3' end of the human growth hormone gene.

**(C)** pMT-HPRT, the mouse HPRT minigene linked to the metallothionein-I

promoter.

**(D)** pSV-HPRT, the HPRT minigene cloned to the SV40 promoter.


Restriction sites: B, Bam H1; Bg, Bgl II; H Hind III; P, Pst I; Pv,

Pvu II; R, Eco RI; S, Sal I; X, Xho I.  Restriction sites with an

asterisk denote artificial sites introduced during the cloning process

(for details see Materials and Methods).

# FIGURE 3.1



A

pDWM1

B

pMT142

C

pMT-HPRT

D

pSV-HPRT
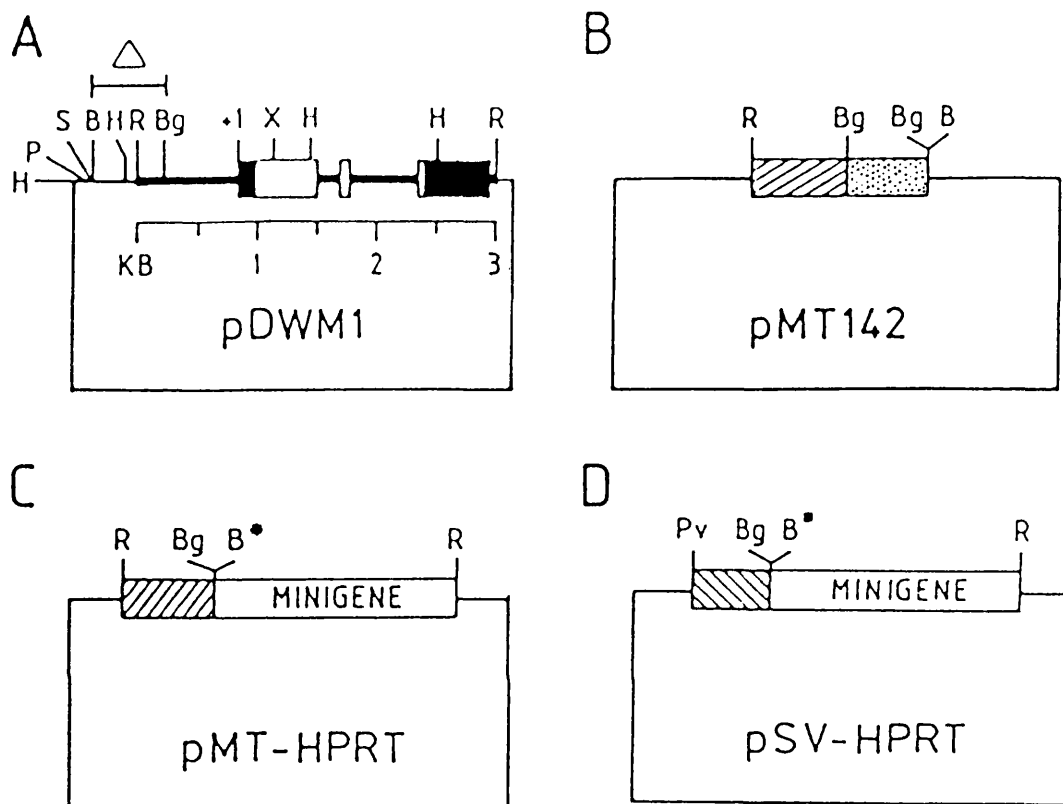
**Figure 3.2** Schematic diagram of steps involved in the experimental procedure. Fertilized 1-cell eggs were injected with different DNA constructs, cultured to 2-cell, 5- to 8-cell and morula and blastocyst stages. Embryo extracts were assayed for HPRT and APRT activities. For details see text.

Figure 3.2



pDWM1, pMT-HPRT, pSV-HPRT

DNA
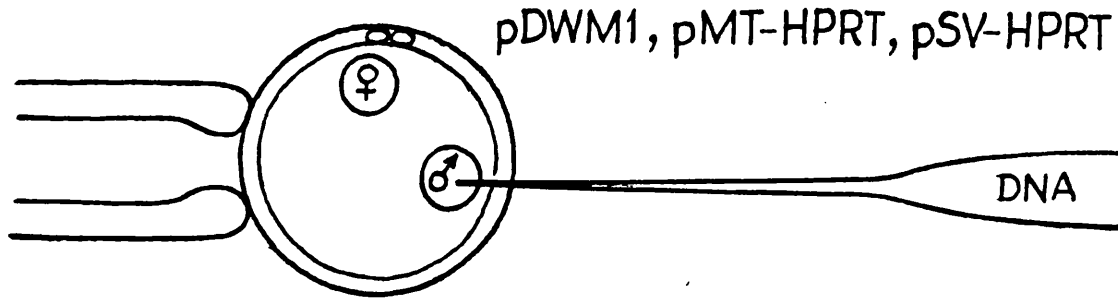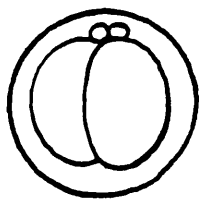
culture to

2c
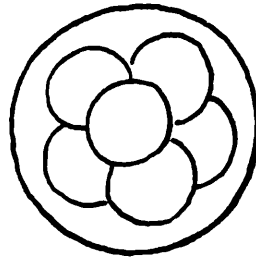
5-8c
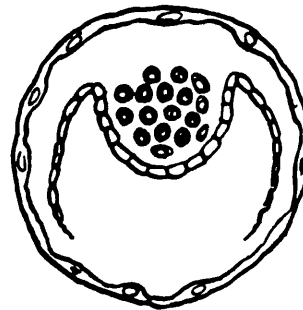
Blastocyst

embryo
extract
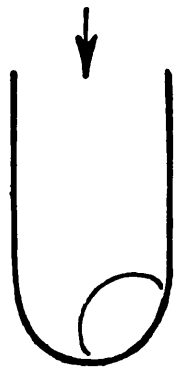
Assay HPRT and APRT

reaction mix

### 3.2.2 Embryo collection and culture

Random bred MF1 (Olac Ltd.) and F1 (CBA X C57BL) females were superovulated (see chapter 1.2.1) and mated with MF1 and F1 males, respectively. Fertilized 1-cell eggs were collected from the oviducts 19-20 hours post hCG injection. After removal of the cumulus cells (chapter 1.2.1) the eggs were washed and transferred to a glass chamber in M2 medium (Fulton & Whittingham,1978) for microinjection (see below). The injected eggs were transferred to droplets of medium 16 and cultured at 37°C as described in chapter 1.2.4. The steps involved in the experimental procedure are shown in figure 3.2.

### 3.2.3. HPRT and APRT microassay

Both injected and control embryos were harvested at 2-cell, 5- to 8-cell and morula and blastocyst stages after, 1, 2 and 3 days of culture, respectively. The embryos were washed in PB1.PVP medium and transferred in 5 μl of the same medium to 10 μl Drummond microcaps. The ends were sealed by melting the glass in a low flame and samples stored at -70°C. Extracts were prepared by freeze-thawing three times and the supernatant assayed for HPRT and APRT as described in chapter 1.2.6. The specific activity of $^3$H-hypoxanthine was either 670 Ci/M, 1625 Ci/M or 1180 Ci/M and of $^{14}$C-adenine, 235 Ci/M. The reactions were incubated for three hours in a 37°C water bath.

### 3.2.4 DNA concentration

The plasmid DNAs were provided by D. Melton. Prior to injection, the plasmid DNAs were diluted to 5 µg/ml in D.H$_2$O and centrifuged at room temperature for 10-15 minutes to pellet any particulate matter that might block the injection pipette. In all the experiments, approximately 2 pl of 5 µg/ml solution of plasmid DNAs (1500-2000 copies) were injected into the male pronucleus of the fertilized egg.

### 3.2.5 Timing of injection

The pronuclei of 1-cell fertilized mouse eggs were injected when they were at maximum size, but before the disappearance of the nuclear membrane. The pronuclei swell progressively during the 1-cell stage and remain prominent for 4-6 hours. The optimal time of the day for microinjection depends upon the strain of mice being used and also depends on the light and dark cycle on which the mice were maintained. We normally switch off the light at 5.30 PM and on at 3.30 AM Under these conditions the eggs can be injected between 3 PM to 8 PM. Fertilized eggs for microinjection experiments were obtained by superovulation of mice as described above.

### 3.2.6 Preparation of microinjection instruments

### (I) Glass Capillaries

15 cm thick walled borosilicate glass capillaries (1 mm outer diameter, GC100 TF-15; Clark Electromedical Instruments) were used for

**Figure 3.3** Diagram showing preparation of a holding pipette for

microinjection.

A-shows the introduction of two bends to allow horizontal use in

an injection chamber

B-shows the breaking of holding pipette at the required diameter

over a heated glass bead

C-shows the correct symmetrical shape of the tip of a holding

pipette, whereas D and E are incorrect. See text for details.

Figure 3.3
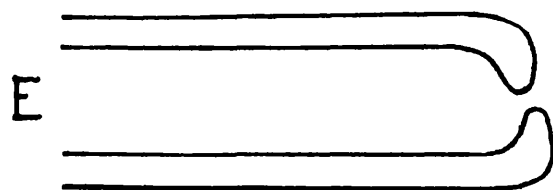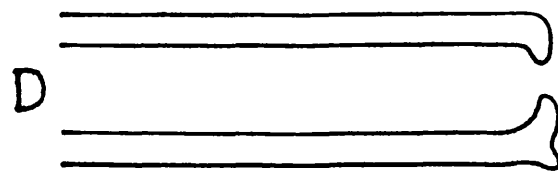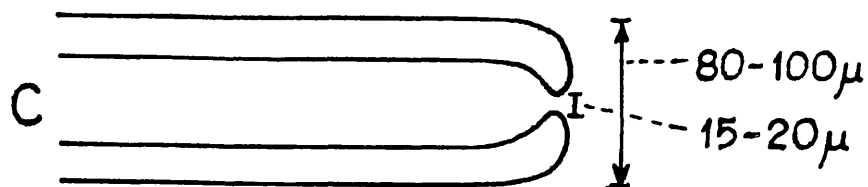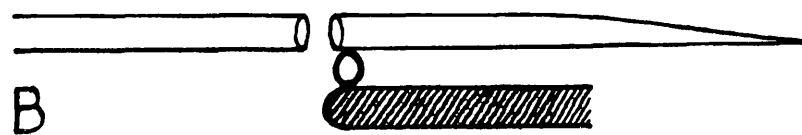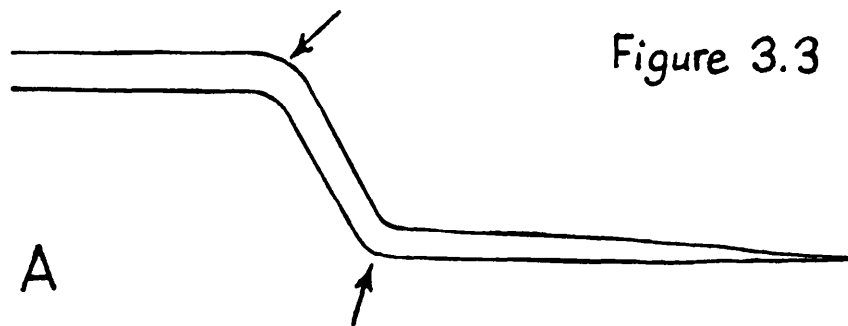
making holding pipettes. 10 cm thin walled capillaries with an
internal fibre (outer diameter 1 mm, internal diameter 0.8 mm, GC100
TF-10; Clark Electromedical Instruments) were used for making
injection pipettes. These capillaries were cleaned by soaking them
overnight in 40% nitric acid and rinsing thoroughly with distilled
water and finally with acetone. In addition, the injection pipettes
were siliconized with dimethyl- chlorosilane solution. The pipettes
were then rinsed in acetone to remove excess silicone and washed
thoroughly with distilled $H_2O$. Both holding and injection pipettes
were baked in the oven overnight at 150°C before use.

## (II) Holding Pipettes

A 15 cm capillary tubing was drawn in a micro-burner to an
outside diameter of 80-100 $\mu$m. If the diameter of the pipette was
larger than required, the desired diameter could be obtained by gently
melting the tube next to the microforge filament (Beaudouin). The
capillary was broken 1.5 - 2 cm away from the narrowing of the tube
and two bends were made over a micro-burner as shown in the figure
3.3A. The introduction of these bends allows the end of the holding
pipette to be positioned horizontally in the injection chamber (figure
3.4A). The capillary tube was then broken neatly at the diameter of
80-100 $\mu$m using the microforge. To do so, the tube was inserted into
an instrument holder and positioned horizontally on the microforge so
that the point at which it reached the required diameter, was just in
contact with the glass bead on the filament. The heated glass bead
was then gently touched to the surface of the capillary tube. When
the two glass surfaces fuse, the heating was switched off immediately.

The sudden contraction of the filament upon cooling broke the glass tube neatly (figure 3.3B). The end of the tube was heat-polished on the hot glass bead until the internal diameter was in the region of 15-20 $\mu$m (figure 3.3C).

## (III) Injection Pipettes.

10 cm siliconized glass capillary tube was pulled on a pipette puller (Camden Instruments Ltd., Model 753) in order to make injection pipettes. A good pipette for injecting mouse eggs should have a tip with a diameter of less than 1 $\mu$m. However, this is below the limits of resolution of the light microscope. Pipettes with orifices that can be visualized under the microscope were rejected since they were considered to be too large. Pipettes with large tips tend to lyse the eggs during microinjection. On the other hand, if the tips were too small, the holes tend to clog easily. The best way to choose a good pipette is to try various pipettes and to use the one that works best. Two needles are obtained from each glass capillary and the tips of the two pipettes are usually similar such that both can be used. To enable the injection pipettes to be positioned horizontally in the injection chamber, two bends were made as shown (figures 3.4B & C), making sure that all the bends were in the same plane. The injection pipettes were usually made on the day of injection since on storage, they usually become blunt and accumulate dirt.

**Figure 3.4 A Injection chamber**

M-medium, HP-holding pipette, CS-cover slip, GS-glass supports,

S-slide, PO-paraffin oil, E-embryos.  See text for details.

**Figure 3.4 B and C**   Preparation of microinjection pipettes

The steps B and C were carried out over a small flame from a

micro-burner to introduce two bends to allow horizontal use in an

injection chamber.

Figure 3.4

**(IV) Injection Chamber.**

The injection chamber is made with a 2x3 inch glass slide onto which four glass supports of 2 mm thickness have been stuck with glue (figure 3.4A). A cover slip (baked) (22x22 mm) was held in place with vacuum grease. A small drop of M2 medium was placed in the centre of the cover slip and the slide below. These two drops are joined together by addition of more medium with the help of a fine pipette forming a continuous column of medium between the slide and the cover slip. The remaining space between the cover slip and the slide was filled with paraffin oil (figure 3.4A). For microinjection, the injection chambers should be clean. They were soaked overnight in 40% nitric acid, rinsed thoroughly with distilled water and finally rinsed twice with acetone, before sterilization in the oven. To clean the chamber after use, the grease and oil were removed with soft tissue and the chambers scrubbed individually with soft a sponge soaked in strong detergent (7X). The chambers were then rinsed thoroughly under running water followed by rinsing in distilled water, so as to remove all traces of detergent, and then oven sterilized.

**3.2.7 The microinjection set-up.**

**(I) Microscope.**

Microinjection can be carried out either using an upright or an inverted microscope. For my microinjection experiments, an upright microscope (Leitz, Laborlux II) was used. This microscope is fitted
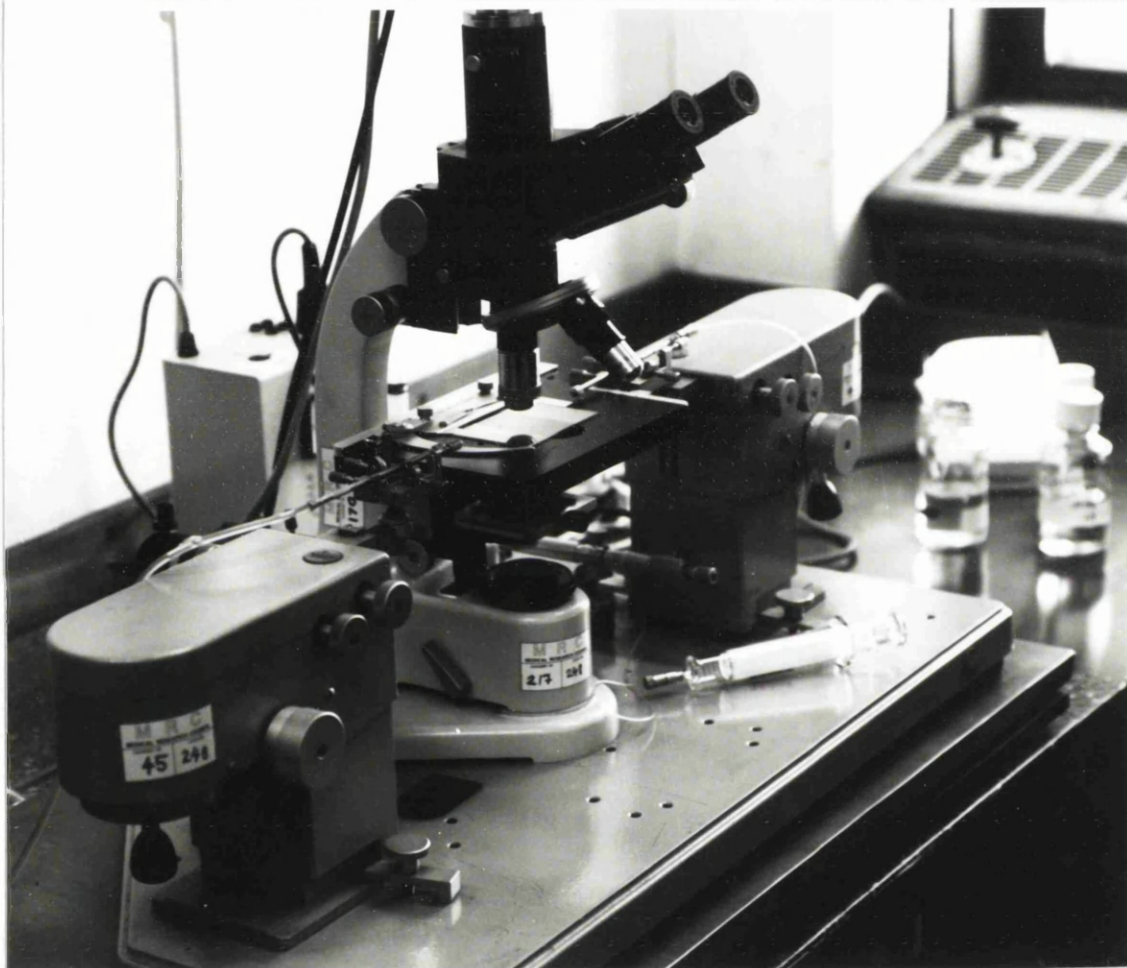
**Figure 3.5** Upright microinjection apparatus.  The microscope

(LaborluxII) manipulators and base plates are made by Leitz.

with objectives of 10X and 16X long working range and 6X wide field eye pieces (figure 3.5).

## (II) Micromanipulators.

Leitz micromanipulators are mounted on each side of the microscope stage. These are controls for the holding pipettes and for the injection pipettes. The "joy-stick" allows simultaneous movement of the pipettes (holding and injection) in two dimensions.

## (III) Table.

Vibration-free conditions are important for microinjection. Building vibration or other sources of vibration (e.g. nearby instruments) may cause the pipette to vibrate when viewed under high magnification. To avoid this problem, a heavy sturdy table was used and positioned away from the wall. In addition, three groups of three squash balls were positioned between the top of the table and a 1 cm thick aluminium plate. A second aluminium plate and a set of squash balls were added to this pile. The microinjection set up was mounted on this. This arrangement reduces the possibilities of any movement of the pipette tip.

## (IV) Micrometer to control holding pipette.

A Leitz instrument tube was connected to a micrometer (a syringe with a mechanism, for fine movement) through a piece of plastic tubing. The syringe was clamped on the right hand side of the

micromanipulator for easy access during microinjection. The entire system was filled with paraffin oil while taking care to eliminate any air bubbles. The entire holding pipette was also filled with paraffin oil and inserted into the instrument tube. The instrument tube with pipette was then clamped on the left hand side of the micromanipulator. The apparatus was then adjusted so that the tip of the holding pipette can reach the injection chamber. Under the microscope, the heavy liquid paraffin should be seen to fill the pipette to its tip.

### (V) Micrometer to control injection pipette.

The tip of the injection pipette was filled with about 1 μl of DNA solution (as described below) and the pipette was inserted into a Leitz instrument tube. The instument tube was connected through a piece of plastic tubing to a 20 ml glass syringe. The entire system was filled with air (except for DNA solution in the tip). The flow of DNA was controlled by the exertion of pressure on the syringe. When pressure was exerted on the syringe, the DNA was expelled out of the tip of the pipette and on releasing the pressure, the plunger returns to its neutral position and the flow of DNA ceases.

### 3.2.8 Pronuclear injection.

(I)    An injection chamber was set up and using a pulled pasteur pipette, several mouse eggs were transferred into the chamber. The assembled holding pipette was inserted into the drop from the left

**Figure 3.6** Microinjection into pronucleus.

A- Incomplete penetration of the egg plasma membrane. The DNA solution expelled from the needle forms a visible bubble but the pronucleus does not swell.  The DNA will flow back out of the egg when the needle is removed.

B- Successful injection of the pronucleus.  The DNA expelled from the needle causes the pronucleus to swell visibly.

Figure 3.6



A

Labels for diagram A: zona pellucida, polar bodies, ♂ pronucleus, membrane indented, DNA 'bubble', ♀ pronucleus, nucleoli

B

Labels for diagram B: polar bodies, ♂ pronucleus, ♀ pronucleus, nucleoli
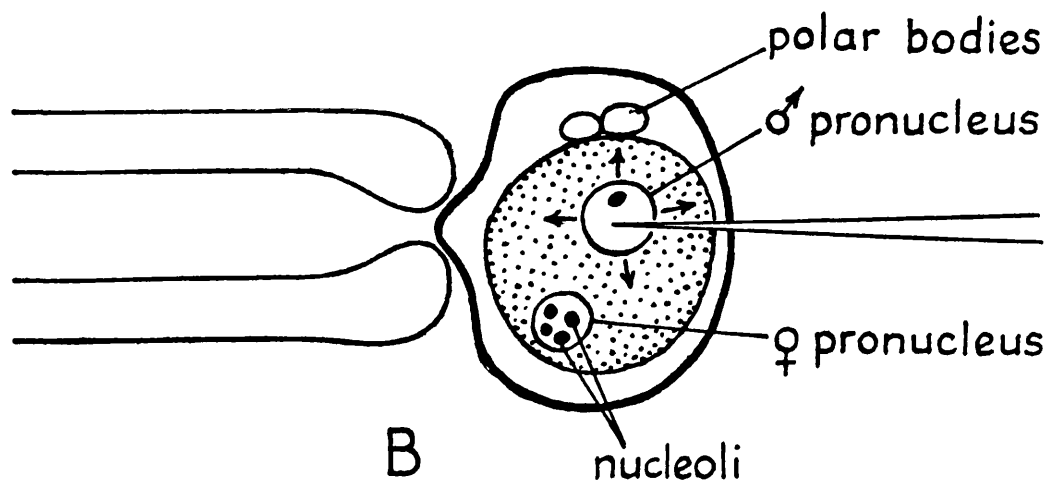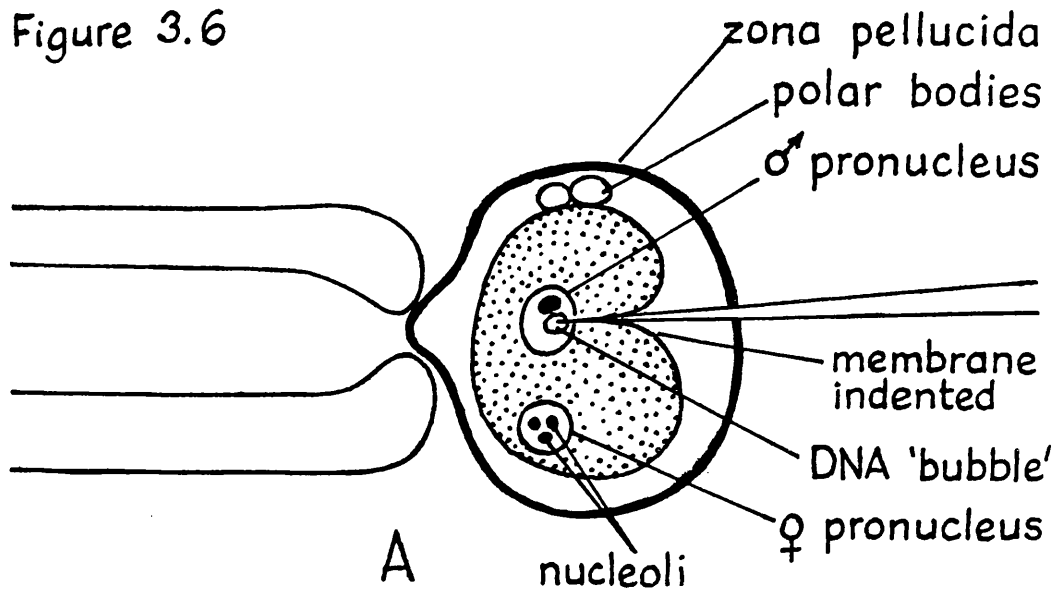
side and adjusted so that it lies horizontally on the bottom of the chamber.

(II)   A siliconized glass capillary was drawn over a flame with a shaft of about 6 cm in length and 0.3 mm in diameter. Using a mouth piece, 2-3 μl of DNA solution was drawn into the glass capillary. The capillary was then inserted into the injection pipette and expelled the DNA at the tip while taking care to avoid any air bubbles. The instrument tube with the injection pipette was clamped to the micromanipulator and the tip of the pipette inserted into the drop of medium.

(III)   It is important to make sure that the injection needle is not closed at the tip or clogged. This can be checked by placing the tip of the pipette next to an egg in the same focal plane and on exerting pressure on the injection syringe, the stream of DNA from the pipette should be able to blow the egg away. If this does not happen, then either the tip of the needle is closed, or it has become clogged. This test is repeated with a new injection pipette.

(IV)   To inject, an egg was oriented with the aid of the the manipulator joy sticks, so that both pronuclei were in focus. A pronucleus can be injected easily if it is located nearest to the injection pipette. Either of the two pronuclei may be injected but the male pronucleus is greater in size which makes it easier to inject than the female pronucleus.

**(V)** By exerting a slight negative pressure on the syringe, the egg is picked up by the holding pipette and when the egg is firmly held,the pronucleus to be injected may be focused by adjusting the manipulator height.

**(VI)** The injection pipette was placed next to the egg and the tip of the needle was brought in focus. The injection pipette was then gently pushed into the pronucleus with the aid of the joy stick. Once the tip of the pipette was inside the nucleus, a positive pressure was applied on the syringe; if the injection is successful, the pronucleus was seen to swell (figure 3.6B). The pronucleus will later shrink back to its original size. A small round bubble was formed around the tip of the pipette if penetration of the plasma membrane was unsuccessful (figure 3.6A).

**(VII)** After injecting the DNA into the pronucleus, the injection pipette was pulled back quickly out of the egg. A slow withdrawal may cause the pronucleus to be pulled out with the pipette.

**(VIII)** The same procedure was repeated for the next egg.

**(IX)** The same injection pipette is used as long as it continues to inject successfully. The problems faced during microinjection are mostly due to blockage of injection pipettes. Clogging of injection pipettes may be due to a variety of reasons, such as high concentration of DNA, the presence of dust in the DNA or nuclear materials which may be picked up from the egg during injection.

(X) When the injection is completed, the healthy eggs which have been operated on are separated from the lysed ones. A healthy egg can be easily distinguished by the presence of a distinct outline and a perivitelline space between the zona pellucida, whereas the lysed egg fills the entire zona. The separated healthy eggs are washed in medium 16 and cultured in the incubator in M16 medium at 37°C.

## 3.3 RESULTS

### 3.3.1 Expression of injected HPRT minigene

The entire HPRT gene is 33kb long and contains 9 exons (Melton et al, 1984). The basic minigene (pDWM1) was constructed by fusing the 5' and 3' ends of the gene onto a fragment of HPRT cDNA. The resulting 3kb minigene functions efficiently in cultured mammalian cells (Melton; 1984). In the experiments reported below, a 5' deletional derivative of the basic minigene, pDWM1Δ-638 was used. pDWM1Δ-638 contains 638 bp of 5' flanking sequence, the entire 5' and 3' untranslated regions and the HPRT coding sequence interrupted by the last two introns (figure 3.1A). Further constructs were prepared (figure 3.1) in which the mouse HPRT promoter sequence from pDWM1Δ-638 was replaced by the promoter sequences of the mouse MT-I gene (pMT-HPRT) or of the Simian Virus SV40 (pSV-HPRT).

The expression of an exogenously introduced HPRT minigene was monitored throughout preimplantation development. The male pronuclei of fertilized F1 (CBA X C57BL) mouse eggs were injected with approximately 2 pl of 5 μg/ml solution of supercoiled plasmids, pDWM1Δ -638 (1500-2000 copies). The injected eggs were collected at the 2-cell stage and assayed for HPRT and APRT. By expressing the results as the ratio of HPRT and APRT activities, the resulting increase in HPRT activity is standardised to the endogenous APRT activity in the embryos. One day after injection with the supercoiled HPRT minigene, the 2-cell embryos showed an approximate twofold increase in HPRT activity, and HPRT:APRT ratio (data not shown), when compared with the

Table 3.1 Expression of HRPT activity from injected and endogenous HPRT genes during preimplantation development

| Days in culture | No. of samples (no.embryos/sample) | Stage of development | [1]HPRT ± SE Injected with pDWM1△-638 | Uninjected |
|---|---|---|---|---|
| 1 | 3(5) | 2-cell | 103 ± 11 | 64 ± 4 |
| 2 | 3(3) | 5-8 cell | 368 ± 21 | 381 ± 23 |
| 3 | 4(1) | morula | 930 ± 203 | 1150 ± 106 |
|  | 1(1) | blocked 1-cell | 7500 |  |
|  | 1(1) | blocked 2-cell | 4420 |  |

[1]HPRT enzyme activity is expressed in femtomoles/hour/embryo
The average APRT activity is similar at all stages in injected and control embryos (data not shown)
Replicate experiments gave similar results (also see table 3.3 under F1 embryo type)

control, uninjected, embryos (table 3.1). The endogenous APRT
activity was similar in uninjected control and injected embryos (data
not shown). The increase in HPRT activity observed in the 2-cell
embryos presumably results from transcription of the injected plasmid
followed by translation into active enzyme protein.

When the embryonic development was continued beyond the 2-cell
stage there was no further increase in HPRT activity and the injected
embryos showed similar HPRT activities to control embryos at the 5- to
8-cell and morula stages (table 3.1). Hence, the injected minigene
DNA may be degraded or inactivated after the 2-cell stage. However,
occasionally, development of the injected embryos remained blocked at
the 1- or 2-cell stage, even after 3 days of culture and, in these
cases, the increase in HPRT activity was most marked (table 3.1).

### 3.3.2 Expression of injected HPRT minigene in 2-cell block embryos

Random bred (MF1) fertilized mouse eggs were injected with HPRT
minigene to investigate whether the high HPRT value would be observed
in embryos blocked spontaneously at the 2-cell stage. When the
morphological development of MF1 embryos was monitored, it was found
that the majority of the embryos cleaved once to the 2-cell stage and
rarely to the 4-cell stage, though they remained viable-looking as 2-
cell (or 4-cell) embryos throughout the 3 days of culture (data not
shown). These MF1 2-cell blocked embryos showed no significant
increase in HPRT or APRT activities throughout the three days of
culture (table 3.2 and figure 3.7).

Table 3.2 Endogenous HPRT and APRT activities in F1 embryos developing to blastocyst and in 2-cell blocked (MF1) embryos

| Embryo type | Days in culture | Stage of development | No of samples[1] | HPRT + SE* | APRT + SE* |
|---|---|---|---|---|---|
| MF1 | 1 | 2-cell | 5 | 142 + 29 | 158 + 32 |
| | 2 | 2-cell | 4 | 255 + 110 | 146 + 45 |
| | 3 | 2-cell | 6 | 151 + 4 | 130 + 13 |
| F1 | 1 | 2-cell | 5 | 132 + 10 | 111 + 3 |
| | 2 | 5-8 cell | 4 | 1318 + 338 | 637 + 61 |
| | 3 | blastocyst | 4 | 2165 + 211 | 764 + 44 |

[1]Single embryo/sample
*HPRT and APRT enzymes are expressed in femtomoles/hour/embryo
The single embryo extracts were incubated for 15 hours at 37°C

**Figure 3.7** HPRT and APRT activities in F1 embryos developing to

blastocyst and in 2-cell blocked (MF1) embryos.

O = HPRT in F1 embryos

Δ = HPRT in MF1 embryos (blocked at 2C)

● = APRT in F1 embryos

▲ = APRT in MF1 embryos (blocked at 2C)

The embryos were harvested singly and assayed for HPRT and

APRT activities.

Figure 3.7
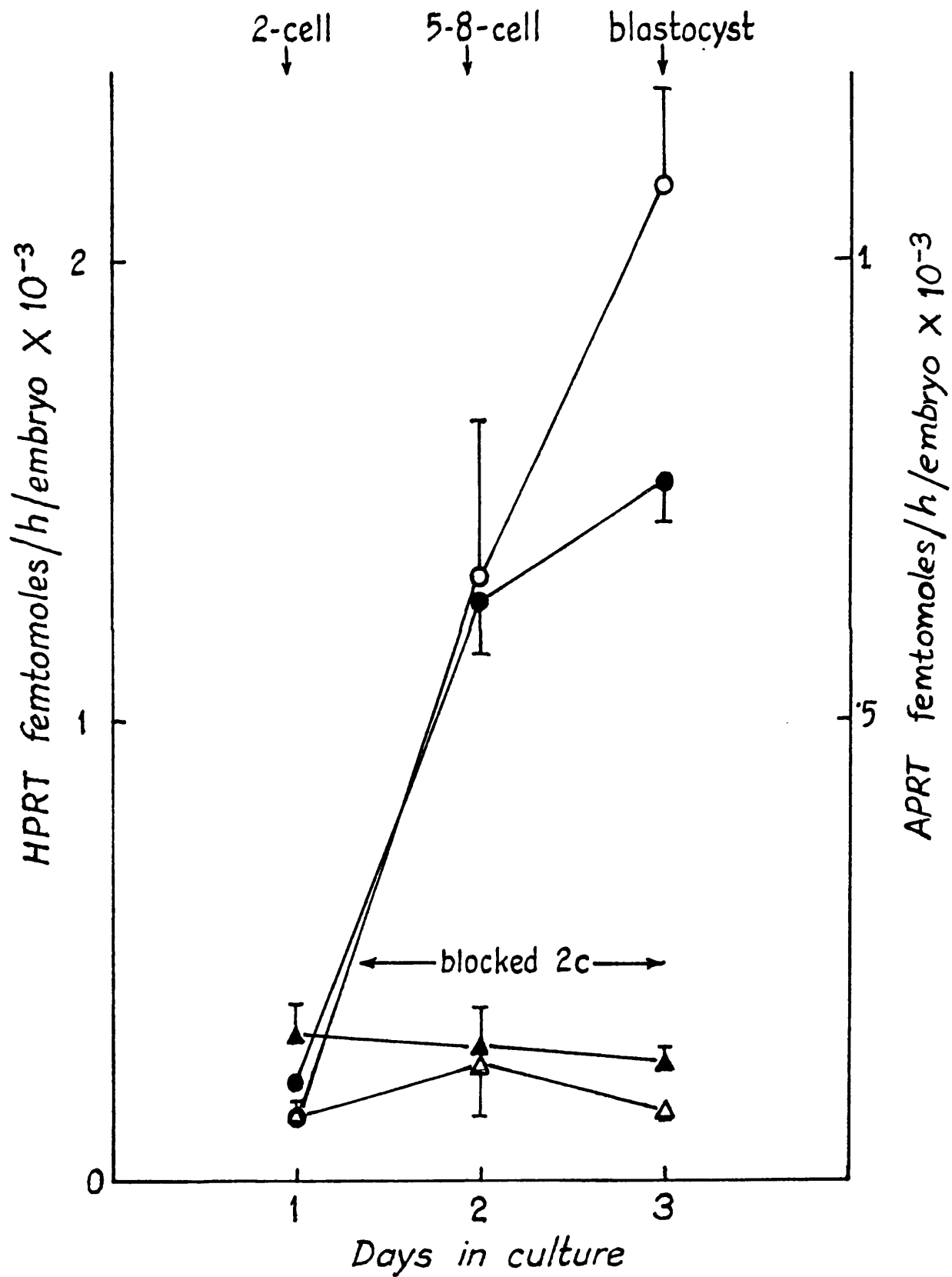
MF1 eggs injected with pDWM1Δ-638 HPRT minigenes showed a twofold increase in HPRT activity compared with the control, uninjected MF1 embryos (table 3.2). However, when the injected MF1 embryos were cultured they remained as blocked 2-cell embryos for 3 days, and showed no further increase in HPRT activity. The approximate doubling of HPRT activity observed in MF1 embryos injected with HPRT minigenes compared with the control uninjected MF1 embryos, after 1 day of culture remained apparent on the third day of culture (table 3.3). As expected, the F1 embryos also showed a significant twofold increase in HPRT activity at the 2-cell stage over control uninjected F1 embryos. However, this increase was not detectable after the 2-cell stage, when the bulk of increased activity is due to the onset of endogenous embryonic gene transcription as the embryos develop to the blastocyst stage.

These results suggest that in 2-cell blocked MF1 embryos (as in F1 embryos), the injected DNA is degraded or inactivated after 1 day of culture, although the translated product already present at the 2-cell stage remains stable for at least 3 days of culture. The quantitative studies by Brinster et al (1976) suggest that most proteins have a long half life. The above results show that activation of the embryonic HPRT gene does not occur in embryos blocked in cleavage divisions. The high HPRT value observed in the blocked F1 (injected) embryos (table 3.1) remains an enigma; it may be due to chance injection of a very high number of plasmid DNAs, resulting into developmental arrest at the 1-cell or 2-cell stage and abundant expression of the injected plasmid.

Table 3.3  Expression of HPRT activity from injected and endogenous HPRT gene in 2-cell blocked (MF1) and F1 embryo

| Embryo type | Days in culture | No of Samples[1] | | Stage of development | [2]HPRT + SE | |
|---|---|---|---|---|---|---|
| | | Injected | Uninjected | | Injected | Uninjected |
| MF1 | 1 | 6 | 5 | 2-cell | 271 + 98 | 142 + 29 |
| | 2 | 5 | 4 | 2-cell | 289 + 43 | 255 + 110 |
| | 3 | 4 | 6 | 2-cell | 307 + 76 | 151 + 4 |
| F1 | 1 | 7 | 5 | 2-cell | 230 + 21 | 132 + 10 |
| | 2 | 4 | 4 | 5-8 cell | 973 + 134 | 1318 + 338 |
| | 3 | 3 | 4 | blastocyst | 2671 + 307 | 2165 + 211 |

[1]Single embryo/sample
[2]HPRT enzyme activity is expressed in femtomoles/hour/embryo
The endogenous APRT activity is similar at all stages in injected and control embryos (also see table 3.2 and figure 3.7)
Replicate experiments gave similar results (data not shown)

### 3.3.3 Promoter fuction

The function of three different promoters was studied in 2-cell mouse embryos using the expression of HPRT as a reporter function. The fertilized eggs were injected with HPRT minigene linked to its proper mouse HPRT promoter, the mouse metallothionein-I promoter or the viral SV40 promoter, and the resulting gene product assayed. The HPRT promoter and the uninduced mouse metallothionein I promoter show an approximate twofold increase in HPRT activity and HPRT:APRT ratio indicating that both promoters function equally well in the early embryo (table 3.4). However, injection of two independently made constructs of the HPRT minigene fused to the SV40 promoter (figure 3.1) into mouse eggs failed to show increased HPRT activity in the embryos at the 2-cell stage (table 3.4). Both pSV-HPRT constructs function in cultured cells (D.W.M., unpublished data). The failure of the pSV-HPRT constructs in the 2-cell mouse embryos is in contrast to expression of gpt (guanosine phophoribosyl transferase) from the SV40 promoter which was observed in 2-cell stage embryos following injection at the 1-cell stage (R.L.B., unpublished data).

### 3.3.4 Chromatin structure of injected plasmid and expression

To investigate the notion that a specific DNA topology is required for transcription (Harland et al, 1983), supercoiled and linearised HPRT plasmid DNAs were injected into the fertilized mouse eggs and the expression of HPRT was compared at the 2-cell stage. For this experiment, the supercoiled pMT-HPRT plasmid DNA was linearised by cleavage of the vector (pUC8) with Bam HI away from the inserted

Table 3.4 Expression of HPRT activity at the 2-cell stage from injected HPRT minigene transcribed from the HPRT or MT-I or pSV promoters

| Injected plasmid | No. of samples (no. embryos/sample) | HPRT* | APRT* | HPRT:APRT |
|---|---|---|---|---|
| pDWM1Δ-638 | 9(1) | 159 + 38 | 158 + 38 | 1.05 + 0.10 |
| pMT-HPRT | 5(1) | 151 + 19 | 120 + 19 | 1.28 + 0.20 |
| pSV-HPRT | 8(1) | 75 + 15 | 137 + 23 | 0.57 + 0.13 |
| Uninjected control | 12(1) | 83 + 11 | 129 + 10 | 0.69 + 0.10 |

*Both HPRT and APRT enzymes are expressed in fentomoles/hour/embryo
Replicate experiment gave similar results (data not shown)

Table 3.5 Expression of HPRT activity at the 2-cell stage from injected
linear and supercoil DNA

| Injected plasmid | No. of samples (no. embryo/ sample | HPRT* | APRT* | HPRT:APRT |
|---|---|---|---|---|
| pMT-HPRT (Supercoil DNA) | 12(1) | 510 + 125 | 125 + 23 | 4.89 + 1.06 |
| pMT-HPRT (Linear DNA) | 19(1) | 257 + 27 | 118 + 10 | 2.30 + 0.26 |
| Uninjected control | 7(1) | 187 + 12 | 115 + 18 | 1.86 + 0.26 |

*Both HPRT and APRT enzymes are expressede in femtomoles/hour/embryo
The experiment was repeated and the results are reproducible (data not shown)

promoter and the HPRT minigene. The use of the same construct (having the same promoter) with different DNA configuration eliminates the possibility of differential HPRT expression from different promoters. When the injected embryos were assayed for resultant HPRT and endogenous APRT activities, the supercoiled DNA showed more efficient HPRT expression and higher HPRT:APRT ratio compared with the linear DNA (table 3.5).

## 3.4 DISCUSSION

### 3.4.1 Expression of injected HPRT minigene throughout preimplantation development

The procedure of injection of the HPRT minigene and detection of resultant HPRT activity by the biochemical double microassay provides a sensitive reporter function applicable to single preimplantation embryos. With this it has been possible to test efficiency of exogenous gene expression from different promoters, from different DNA conformations and under different conditions of development throughout preimplantation mouse embryo development. The simultaneous assay of endogenous APRT activity provides an internal standard which enables accurate comparison to be made between experimental and control values.

Some variability in HPRT levels may be observed in control embryos from one experiment to another. This variability is always seen in this highly sensitive microassay and possible reasons are explained in chapter 1 (section 1.3.1). However, all of the comparisons have been done within the same experiment and results are internally consistent from one experiment to another. The variability does not affect the interpretation and the conclusions drawn from the experiments.

The HPRT activity from the exogenously introduced gene is observed as an increase in activity over and above the endogenous HPRT activity levels. The increase is attributable to an increase in HPRT

enzyme activity transcribed from the injected plasmid DNA and translated into detectable active enzyme. Endogenous HPRT and APRT activities are low in the egg and an increase is observed throughout preimplantation development. The increase in HPRT activity up to 4-cell stage is coded by stored maternal mRNA but thereafter a more rapid increase is governed by the expression of the embryonic gene (Harper & Monk, 1983; also see chapter 1). The twofold increase in HPRT activity above the endogenous level following injection of the HPRT minigene is transient and there is no further increase beyond the 2-cell stage (tables 3.1 and 3.3). The reason for the cessation of synthesis of new enzyme from the injected plasmid may be that the injected DNA is degraded or inactivated beyond the 2-cell stage (table 3.1).

In rare cases, when the injected embryos remain blocked at the 1-cell or 2-cell stage, they show a marked increase in HPRT activity (table 3.1) even after three days of culture. A similar large increase in E. coli gpt activity in embryos injected with cloned gpt sequences, and subsequently blocked in development, has been previously observed (R.L.B., unpublished). To determine the reason for high gene activity in blocked F1 embryos, MF1 embryos, which normally become blocked at the 2-cell stage were injected with HPRT minigene and the HPRT activity was compared with injected F1 embryos up to 3 days of culture. The control, uninjected, MF1 embryos showed no excess synthesis of endogenous HPRT and APRT activities when cultured for three days (table 3.2 and figure 3.7). The injected MF1 embryos showed higher HPRT activity over the uninjected control embryos after 24 hours of culture. However, when cultured for three

days, they showed no excess increase in HPRT activity above the
initial increase after one day of culture even though the embryos
remained blocked at the 2-cell stage. Interestingly, the initial
increase (after one day of culture) remain stable even after three
days of culture. These results suggest that the increased enzyme
activity observed from the injected DNA is not degraded at least for
three days in culture. Similar stability of both endogenous HPRT and
APRT activity is observed in control uninjected MF1 embryos blocked at
the 2-cell stage (figure 3.7 & table 3.2). A similar prolonged
stability of HPRT and APRT activities in unfertilized mouse eggs (see
chapter 1), and of the HPRT activity in unfertilized human eggs has
been observed (Braude et al, 1989)

Since the increased expression of DNA injected into the
fertilized eggs may be observed clearly at the 2-cell stage (table
3.1), all the manipulated embryos were subsequently monitored for gene
activity at this stage.

## 3.4.2 Promoter function in mouse preimplantation embryos

The HPRT and APRT microassay was used to study promoter function
in mouse embryos. The results showed successful translation of HPRT
gene from the mouse proper HPRT promoter and the mouse uninduced
metallothionein-I promoter, whereas the viral SV40 promoter did not
allow HPRT expression. However, the same construct (pSV-HPRT) was
expressed in cultured cells. The studies on the expression of SV40
genes in mouse oocytes showed that both cis- and trans-acting
sequences are required for successful gene expression (Chalifour et

al, 1987). Recent studies by Gorman et al (1985) showed efficient SV40 gene expression in undifferentiated embryonal carcinoma (EC) cells when transfected with large amounts of DNA. However, in another cell transfection study with embryo-derived stem cells (ES) and F9 cells, the SV40 promoter linked to an HPRT gene did not give any HAT resistant colonies, whereas the same promoter linked to reporter gene neo was able to produce a small number of G418 resistant colonies (Marianne Frommer, personal communication). It is possible that the specific sequences linked to the SV40 promoter actually affect the function of the promoter itself. This type of phenomenon has also been observed with other promoters e.g., the mouse MT-I promoter. When the mouse MT-I promoter was fused to the structural gene of growth hormone, the levels of mRNA were high but when the same promoter was fused to TK gene, the mRNA level was very low (Palmiter et al, 1983b). Thus, these results suggest that different structural genes may influence the rate of transcription and the gene expression in an unexplained way. In the experiments reported here, it is not known whether the failure of the SV40 promoter to give HPRT activity in embryos occurs at the level of transcription or translation.

### 3.4.3 Chromatin structure of plasmid and expression

Supercoiling of DNA in prokaryotes is an important requirement for proper gene function (reviewed by Gellert, 1981; Smith, 1981). In eukaryotes, such a mechanism was viewed as unlikely since most of the chromosomal DNA, which is usually transcriptionally silent is not under superhelical tension (Sinden et al, 1980). However, recent studies showed that most deoxyribonuclease I hypersensitive regions

contain specific sites that are sensitive to enzymes like S1 nuclease and chemicals such as bromoacetaldehyde, which detect non-B form DNA (Larsen & Weintraub, 1982; Kohwi-Shigematsu et al, 1983). Many of these sites are seen in supercoiled but not in relaxed DNA.

Several studies suggest that supercoiled DNA is more efficient in transcription compared with linear DNA (Harland et al, 1983; Sollner-Webb & Mcknight, 1982; Pruitt & Reeder, 1984a,b) and DNA supercoiling may be required for gene transcription in vivo (Ryoji & Worcel, 1984). Recently, Weintraub et al (1986) compared CAT gene expression in cell culture from transfected DNAs of different topologies and showed that supercoiled DNAs are over 100-fold more effective as templates than linear DNAs.

The experiment presented in this study showed higher HPRT activity from supercoiled DNA compared with linear DNA. The supercoiled DNA shows an approximate twofold increase in HPRT:APRT ratio over linear DNA. A similarly high TK gene transcription was observed from supercoiled DNA when injected into Xenopus oocytes. The same plasmid DNA when linearised showed reduced transcription (Harland et al, 1983). The lower HPRT gene activity from the linear molecule may be due to inefficient transcription of the gene in vivo (Pruitt & Reeder, 1984a; Harland et al, 1983). However, from our experiments it is difficult to conclude whether the different levels of gene expression from the two conformations of DNA molecules occur at the level of transcription or translation. The possibility that linear molecules are degraded by exonucleolytic attack (Wyllie et al, 1978) has been ruled out and no differential stability of the two forms of

templates could be detected (Harland et al, 1983; Pruitt & Reeder, 1984a). The true reason as to why a supercoiled template may be required for successful transcription and gene expression is not known. Supercoiling may be needed for transducing signals from one region of the chromosome to another (see review by Weintraub, 1985). Supercoiling or a closed topology may also increase the bending of DNA and facilitate interaction of distantly placed, cis-acting regulatory sites (Dunn et al, 1984).

The results obtained from these studies provide background information important for experiments concerned with manipulation of endogenous and exogenous gene expression during mouse preimplantation development. When a plasmid carrying HPRT minigene was injected into the fertilized mouse eggs, a twofold increase in HPRT activity and HPRT:APRT ratio was observed at the 2-cell stage. This increase was not detected beyond the 2-cell stage, therefore all the manipulated embryos were subsequently monitored for gene activity at this stage. The results on different promoter function indicates that mouse HPRT and MT-I promoter function equally well in early cleavage stages of mouse embryos where as SV40 does not allow HPRT expression. Furthermore, the study of different DNA conformations showed higher HPRT activity and HPRT:APRT ratio from supercoiled DNA compared with linear DNA.

# CHAPTER 4

## Manipulation of HPRT minigene expression in preimplantation mouse embryos

## 4.1 INTRODUCTION - Inducible promoters and antisense DNA and RNA

The possibility of manipulating gene expression in early mammalian embryogenesis is important for the study of the genetic programme in development. One approach would be the use of inducible promoters such as those directing the expression of mouse mammary tumour virus (MMTV) genes (Holt et al, 1986), the interferon (Goodbourn et al, 1985) and heat-shock protein genes (Wu, 1984; Topol et al, 1985), and the metallothionein gene (MT) (Mayo et al, 1982). The expression of a specific gene product may also be manipulated by selectively inhibiting its messenger RNA with complementary antisense RNA. In this case, the formation of a stable duplex between messenger (sense) RNA and its complementary antisense RNA results in an inhibition of translation of the mRNA (for review, see Green et al, 1986 and Weintraub et al, 1985). An alternative strategy employing antisense oligodeoxynucleotides has been used in parallel with antisense RNA studies and has shown a similar potential for inhibition of gene expression in eukaryotic cells. Antisense oligonucleotide inhibition of gene expression has been shown in reticulocyte lysate or wheat germ cell systems (Minshull & Hunt, 1986; Cazenave et al, 1987) and in Xenopus oocytes and eggs (Kawasaki, 1985; Cazenave et al, 1987; Shuttleworth & Colman, 1988). These studies indicate that antisense oligonucleotides operate primarily by forming a DNA-RNA hetero-duplex which results in RNase H cleavage of the mRNA (Minshull & Hunt, 1986).

In the following sections of this introduction I review examples of inducible promoters and the role of antisense DNA and RNA in the

regulation of gene expression in prokaryotic and eukaryotic systems. The experiments to be presented in this chapter make use of the metallothionein promoter and antisense DNA constructs to induce and negate HPRT minigene expression in the preimplantation embryo.

### 4.1.1 Inducible Metallothionein promoters

Metallothioneins (MT) are small cysteine-rich proteins of vertebrates and invertebrates that bind heavy metals and are thought to be involved in zinc homeostasis and resistance to heavy metal toxicity (Cherian & Goyer, 1978). All vertebrates examined contain two or more distinct MT isoforms, which are grouped in two classes, designated MT-I and MT-II. In the mouse the two forms of MT differ slightly in amino acid composition but have the same metal-binding capacity (Durnam & Palmiter, 1981; also see review by Hamer, 1986). These two genes are tightly linked, with the MT-I gene lying 6 kb downstream from the MT-II gene and oriented in the same direction of transcription (Searle et al, 1984).

MT synthesis in many different mammals and most other higher eukaryotes is inducible by a variety of heavy metal ions including cadmium, zinc and copper (Durnam & Palmiter, 1981; Cherian & Gover, 1987). Several reports have shown that the levels of hepatic, renal and intestinal MT synthesis increase following the administration of of heavy metals (Cherian & Goyer, 1978; Richards & Cousins, 1977; Durnam & Palmiter, 1981). Maximal rates of the MT synthesis (measured by [$^{35}$S]-cysteine incorporation) are seen 3 to 10 hours after metal administration (Probst et al, 1977; Durnam & Palmiter, 1981).

Induction occurs at the level of transcription. A 2- to 4-fold increase in functional MT mRNA levels in liver results from the administration of cadmium or zinc (Squibb & Counsins, 1977; Shapiro et al, 1978). Actinomycin D inhibits the induction of translatable hepatic MT mRNA if it is given prior to metal administration (Shapiro et al, 1978) further suggesting that the gene is regulated at the transcriptional level (Shapiro et al, 1978; Durnam & Palmiter, 1981)

MT synthesis is also induced by glucocorticoid hormones (Hager & Palmiter, 1981; Hamer 1986) and bacterial endotoxin (Durnam et al, 1984). Direct evidence for a role of glucocorticoids in regulating MT levels was obtained in primary rat hepatocyte culture (Failla & Cousins, 1978). Glucocorticoid induction has also been observed at varying levels, in cultured fibroblast and hepatoma, sarcoma and erythroid cells (Mayo & Palmiter, 1981). Further experiments on nuclei isolated from mouse liver again showed that induction occurs primarily at the level of transcription (Hager & Palmiter, 1981). However, the mouse pMT-I fusion genes are not transcriptionally regulated by glucocorticoids when introduced to mammalian cell sytem (Mayo et al, 1982; Pavlakis & Hamer, 1983) or in transgenic mice (Palmiter et al, 1982). The reason for this is not known.

The inducibility of the mouse MT-I promoter by cadmium has been shown in several experimental systems. When the mouse MT-I gene was transfected into human HeLa or mouse L cells, an increased level of MT mRNA was observed upon cadmium induction (Mayo et al, 1982). Similarly, introduction of a mouse pMT-human growth hormone gene construct, using a bovine papilloma virus vector, into mouse cells

showed induced expression of the hybrid gene by cadmium (Pavlakis et al, 1983). The induction of the mouse MT-I promoter was also demonstrated in early mouse embryos. In this case, the mouse pMT-TK (thymidine kinase) fusion gene was regulated by cadmium following microinjection into the male pronucleus of a fertilized 1-cell mouse egg (Brinster et al, 1982).

The development of transgenic mice with an integrated construct driven by the inducible MT promoter enabled the controlled regulation of gene expression in an animal model system. To obtain transgenic mice, eggs were microinjected either with pMT-TK (Brinster et al, 1981), pMT-rat-growth-hormone gene (Palmiter et al, 1982b) or pMT-human-growth-hormone gene (Palmiter, 1983a). The expression of the mouse pMT-fusion genes in all these transgenic mice was inducible by heavy metals and bacterial endotoxin but not by glucocorticoids (Palmiter et al, 1982a,b; Palmiter et al, 1983a; Durnam et al, 1984).

The specific region(s) of the mouse MT-I gene promoter necessary for regulation has been shown by fusing deleted variants of the MT-I promoter to assayable genes, such as the viral TK gene. The fused genes were then introduced into either tissue culture cells or mouse eggs and metal regulation was analysed (Brinster et al, 1982; Mayo et al, 1982). These studies indicate that the region of the mouse MT-I promoter necessary for metal regulation maps within 90 nucleotides 5' of the normal transcription initiation site of the MT-I metallothionein mRNA. A comparison of the induction of the mouse MT-I and MT-II genes by different metals showed identical dose-response curves for each ion tested both in whole mice (Searle et al, 1984) and

in cultured cells (Yagle & Palmiter, 1985). In contrast, a comparison
of human MT-I to MT-II showed that the MT-II gene responded equally
well to cadmium and zinc whereas the MT-I gene was strongly inducible
only by cadmium (Richards et al, 1984). Transfection experiments
using human pMT-viral TK fusion constructs demonstrated that this
differential regulation is controlled by the 5' flanking region of
each gene (Richards et al, 1984).

## 4.1.2 Other inducible promoters

In addition to metallothionein promoters, there are other
promoters which can be induced by different inducing agents. Human $\beta$-
interferon gene expression can be induced by Sendai virus in mouse
fibroblasts (Goodbourn et al, 1985). Mouse mammary tumour virus
(MMTV) promoter is found to be inducible by steroids in mammalian cell
lines (Holt et al, 1986; Izant & Weintraub, 1985). The MMTV promoter
fused to the CAT (chloramphenicol acetyl transferase) gene showed 20-
fold increase in CAT activity when transfected to mouse cell lines in
the presence of dexamethasone (Holt et al, 1986). Another example is
the promoter of the heat-shock protein gene which is induced by
exposure to high temperature (McGarry & Linquist, 1986).

All the inducible promoters mentioned above may be suitable for
the manipulation of gene expression depending on the system to be
studied. The mouse MT-I promoter was used in the following
experiments because its inducibility by cadmium in the early embryo
had been established (Brinster et al, 1982; Stuart et al, 1984).

## 4.1.3 Antisense RNA

In both prokaryotes and eukaryotes, specific gene expression may be controlled by the protein products of other regulatory genes which act as activators or repressors. Recently it has been discovered that gene regulation also may be mediated by the synthesis of specific RNA sequences. The regulatory RNA is complementary to the messenger RNA of a particular gene. A double stranded RNA is formed by base pairing between these two strands of RNA resulting in an inhibition of mRNA translation (see section 4.4.3). These regulatory RNAs are known as antisense RNA and the DNA that directs the synthesis of antisense RNA is called antisense DNA.

Antisense RNA regulation was initially discovered in prokaryotes (Mizuno et al; 1983, Mizuno et al, 1984, Simon & Kleckner, 1983). In eukaryotes, naturally occurring antisense RNA has not been identified as yet.

Following the finding that antisense RNA can inhibit gene expression in natural systems, strategies were developed to artificially regulate gene expression using antisense RNA. A vector can be designed to synthesize, in vivo, antisense RNA complementary to a target mRNA to inhibit the expression of the respective gene. Both in prokaryotes and in eukaryotes, the function of endogenous genes has been suppressed by artificial antisense DNA or RNA. In eukaryotic cells, specific inhibition of gene expression has been observed by direct microinjection of antisense RNA, synthesized in vitro. The

successful manipulation of gene expression by antisense RNA in transgenic systems shows its potential use in basic and applied research. The current knowledge of the role of antisense RNA in natural and experimental systems is reviewed below.

## 4.1.4 Antisense RNA in prokaryotic systems

The study of plasmid replication in E. coli first indicated a regulatory role of small complementary RNAs. The replication of members of the ColE1 and FII plasmid groups, is negatively controlled by a distinct untranslated RNA species around 100 nucleotides long (Tomizawa & Itoh, 1981; Rosen et al, 1981). These small RNAs, apart from regulating plasmid copy number, prohibit the stable maintenance of two similar plasmids in the same cell, a phenomenon known as incompatibility. In a similar way the replication of a Staphylococcus aureus plasmid, pT181, was shown to be regulated by two small RNAs (Kumar & Novick, 1985).

Detailed analysis of the transposon, Tn10, has revealed that the activity of this element can also come under antisense RNA control. Simon & Kleckner (1983) provided evidence that the insertion sequence, IS10, the active element in transposon Tn10, can negatively control expression of its own transposase protein at the translational level.

Antisense RNAs are naturally encoded by bacterial and phage genomes. It was reported that a small RNA transcript of 174 bases was produced upon growing E. coli cells in high osmolarity, which inhibits the expression of the gene for an outer membrane protein (OmpF

protein; Mizuno et al, 1984). The inhibition of OmpF protein

production by the small RNA transcript (micRNA; mRNA-interfering

complementary RNA) was thought to be due to the formation of a hybrid

between the micRNA and the OmpF mRNA over a region of approximately 80

bases, including the ribosome binding site and the initiation codon

(Mizuno et al, 1983, 1984). The discovery of micF RNA prompted the

development of a system designed to artificially regulate the

expression of any specific gene in E. coli with antisense RNA (Coleman

et al, 1984). Induction of a plasmid that produces complementary RNA

to the E.coli lpp mRNA efficiently blocked lipoprotein production and

reduced the amount of lpp mRNA. A similar inhibition of OmpC and $\beta$-

galactosidase production was achieved using a construct which

generates RNA complementary to OmpC or $\beta$-galactosidase mRNA

respectively (Coleman et al, 1984; Pestka et al, 1984).


Antisense RNA also seems to be one of the regulating factors

involved in phage $\lambda$ development. A promoter, designated $P_{aQ}$ has been

shown to direct antisense transcription of part or all of the amino-

terminal region of the $\lambda$ Q gene encoding sequence (Hoopes & McClure,

1985). Q protein is the antiterminator required for $\lambda$ late gene

transcription. The antisense transcript is thought to repress the

synthesis of the Q protein by interfering with Q transcription or

translation (Hoopes & McClure, 1985). Another regulatory system

involving a small antisense RNA has been identified in the Salmonella

phage P22 (Wu et al, 1984; also see review by Green et al, 1986).

## 4.1.5 Antisense in eukaryotic systems

### I Cell culture systems

The capacity of nucleic acid to inhibit specific gene expression in eukaryotes was initially suggested by the early work with Rous Sarcoma Virus (RSV). Zamecnik & Stephenson (1978) and Stephenson & Zamecnik (1978) showed that synthetic single-stranded oligodeoxynucleotides, complementary to RSV terminal repeats, can diminish normal viral infection and inhibit viral RNA translation <u>in vitro</u>.

The first experimental demonstration of regulation of gene expression by artificial antisense RNA in eukaryotes was shown in a mouse cell culture system (Izant & Weintraub, 1984). For these studies, the Herpes Simplex Virus thymidine kinase gene (HSV-TK) was cloned into a plasmid vector. Transcription of the gene was directed by the HSV-TK promoter and the transcript linked to SV40 polyadenylation signals. The antisense DNA construct was identical to the sense construct except that the DNA sequence of the TK structural gene was inverted relative to the promoter and the poly A site. Thus, transcription of the antisense gene would produce RNA complementary to the normal TK mRNA. A mixture of both sense and antisense HSV-TK plasmids were microinjected into mouse LTK⁻ cells at a ratio of 1:100, respectively. This resulted in a significantly lower level of transient TK expression compared to the cells that were injected with only sense DNA. Further studies with antisense TK genes tested the sequence specificity of this type of inhibition (Izant & Weintraub,

1985). The anti-HSV-TK inhibited the expression of the HSV-TK gene in mouse LTK⁻ cells but had no effect on the expression of the chicken-TK gene. The expression of chicken-TK construct was specifically inhibited only by anti-chicken-TK construct which in turn did not alter HSV-TK gene expression. These studies also showed that antisense transcripts as short as 52 bases complementary to the 5' untranslated region of the TK mRNA were enough to inhibit the TK expression.

In parallel to microinjection studies, antisense work has been carried out by transfection in cell culture systems. Co-transfection of LTK⁻ cells with sense and antisense genes was found to effectively reduce TK⁺ colony formation on selective medium (Izant & Weintraub, 1985). Other transfection studies showed that gene expression of several exogenous (TK, CAT) and endogenous (actin, TK, heat shock-protein) genes could be inhibited in the cell culture system (Izant & Weintraub, 1984; Kim & Wold, 1985; McGarry & Lindquist, 1988).

## II Oocytes and developing systems

The use of antisense RNA can also be extended to study the function of a gene product of interest and its role in early development. Despite the encouraging results in in vitro systems, it was not known whether a DNA vector directing the synthesis of a antisense RNA could be used to study gene function during embryogenesis. To synthesize sufficient quantity of specific antisense RNA, a DNA vector must have a strong promoter that works efficiently in the system used. Morever, a problem arises when one is

interested in studying the function of a previously transcribed gene or genes in cells that are not transcriptionally active in early development. During early development, many of the new gene products which may be important for early determinative events are produced by translation of stored maternal mRNA.

With these limitations in mind, Melton (1985) explored an alternative method for supplying antisense RNA. The method involved synthesizing antisense RNA in vitro with SP6 RNA polymerase, capping in vitro and microinjecting this antisense RNA into the cytoplasm. The first such experiments involved direct microinjection of antisense β-globin RNA into frog oocytes (Melton, 1985). Antisense RNAs complementary to various regions of globin message were injected into Xenopus oocytes which were incubated for a number of hours before a second injection with globin mRNA. Specific inhibition of globin production was observed while the translation of unrelated messages was unaffected. The inhibition of globin synthesis occured only when the antisense RNA included the sequences complementary to the 5' end or the region encompassing the translation initiation site of the globin message. No inhibition of globin production was observed with antisense RNAs complementary to the 3' translated or untranslated region of the message. When RNA was extracted from oocytes and digested with single-strand specific ribonucleases, the protected duplex RNA was found only in cells containing both globin mRNA and antisense RNA indicating that hybridization does occur in vivo. Similar results have been obtained for RNA microinjection experiments with TK and CAT RNA and assay for TK and CAT enzymes in Xenopus oocytes (Harland & Weintraub, 1985).

In a different experiment, CAT activity expressed from a plasmid DNA transcribed into RNA in the oocyte nucleus was inhibited by antisense RNA injected into the oocyte cytoplasm (Harland & Weintraub, 1985). Further studies in Xenopus oocytes showed successful inhibition of acetylcholine receptor of Torpedo electroplaques with antisense RNA (Sumikawa & Miledi, 1988).

Endogenous gene expression for membrane skeleton protein 4.1 was also successfully inhibited by antisense RNA in developing Xenopus embryos. Plasmids expressing antisense 4.1 RNA were microinjected into fertilized Xenopus eggs. Nuclease protection assays revealed that antisense protein 4.1 RNA leads to the specific loss of endogenous protein 4.1 transcripts after midblastula transition (Giebelhaus et al, 1988). Previously, inhibition of gene expression by injection of antisense RNA was successful in oocytes (Wormington, 1986; Melton, 1985) but not in developing embryos due to an RNA duplex unwinding activity (Rebagliati & Melton, 1987; Bass & Weintraub, 1987). However, this activity was very much diminished in late blastula embryos (Rebagliati & Melton, 1987; Bass & Weintraub, 1987). The successful inhibition of protein 4.1 in developing embryos may have occurred because the antisense transcripts for this protein are transcribed from the plasmid only after the mid blastula transition.

Manipulation of gene expression by antisense RNA was applied to a mammalian developmental system, the preimplantation mouse embryo (Bevilacqua et al, 1988). Activity of $\beta$-glucuronidase activity increases 60-fold from the 4-cell to the blastocyst stage during preimplantation development of mouse embryos due to a 13-fold increase

in $\beta$-glucuronidase mRNA. Injection of antisense RNA, produced in vitro from an antisense $\beta$-glucuronidase DNA, into the cytoplasm of a fertilized 1-cell egg, or into each blastomere of the 4-cell embryo results in 45% and 65% inhibition, respectively, of endogenous $\beta$-glucuronidase activity by the blastocyst stage. Capped antisense RNAs injected at the 4-cell stage gave increased inhibition (75%) of $\beta$-glucuronidase expression. Thus, 5' capping may be important for message stability in mouse embryos (Bevilacqua et al, 1988). In addition, there was no evidence of RNA unwinding activity in early mouse embryos as observed in Xenopus embryos (Rebagliati & Melton, 1987; Bass & Weintraub, 1987).

Introduction of antisense RNA into developing systems has also been described in other organisms. The Krüppel (Kr) gene, which is transcribed and expressed at a certain stage in Drosophila development, is known to play a role in controlling segmentation of the embryo. Wild type Drosophila embryos injected with antisense Kr RNA were found to develop Kr phenotypes at a high frequency (Rosenberg et al, 1985). Another example of mutant phenotype (phenocopy) induced by antisense RNA was observed in Dictyostelium, a cellular slime mould (Crowley et al, 1985).

## III Transgenic systems

Antisense RNA has been successfully used to manipulate gene expression in transgenic individuals in several phylla. In the mouse, during the course of my work, it was reported that conversion of normal behaviour to a phenocopy of the shiverer mutant can be effected

by inhibition of the synthesis of myelin basic protein (MBP) by
antisense MBP DNA integrated into the chromosomes of transgenic
animals (Katsuki et al, 1988). In plants, integrated antisense DNA
constructs have resulted in inhibition of expression of the
polygalacturonase gene in transgenic tomatoes (Smith et al, 1988) and
inhibition of flower pigmentation in transgenic Petunia hybrida (Van
der Krol et al, 1988). Antisense technology can also be used to
engineer virus resistant plants and may provide in the future the
potential selective control of gene expression in specific tissues or
at specific stages in development with appropriately regulated
promoters.

## 4.1.6 Aims of the experiments in this chapter

Knowing that the MT-I promoter can be induced by cadmium, a
plasmid constructed with the HPRT minigene was linked to the mouse MT-
I promoter (pMT-HPRT). We aim to establish an inducible system by
injecting this plasmid into mouse eggs followed by induction of HPRT
enzyme activity in the cleavage stages of the embryo. In contrast, we
attempt to inhibit enzyme expression from the injected HPRT minigene
by simultaneous injection of antisense DNA. In previous experiments
large excess of antisense DNA or RNA were required to achieve
inhibition of gene expression. Here, we investigate whether a pMT-
antiHPRT construct can be induced by heavy metals to achieve
repression of HPRT gene activity with equivalent molar amounts of
antisense to sense molecules in early mouse embryos. Once the
efficacy of these manipulative techniques is established in early
embryos, transgenic mice can be made in order to study stage and

tissue specific effects of an inducible HPRT antisense construct on endogenous HPRT gene expression.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Microinjection of embryos

Female F1 (CBA X C57BL) mice were superovulated (see chapter 1.2.1) and mated with F1 males to produce fertilized eggs. One cell zygotes were collected from the oviducts 19-22 hours post hCG injection and the cumulus cells removed by treatment for 5-7 minutes with hyaluronidase (see chapter 1.2.1). The eggs were washed and transferred to a glass chamber (see chapter 3.2.2) in M2 medium. The paternal pronuclei of the eggs were injected with 5 $\mu$g/ml of supercoiled plasmid DNA unless otherwise stated (see chapter 3; Materials and Methods). The injected eggs were transferred to droplets of M16 medium under paraffin oil and cultured at 37°C in a 5% $CO_2$-in-air atmosphere.

### 4.2.2 Cadmium treatment

A stock solution of cadmium sulphate (Sigma) (1 mM) was aliquoted in 1 ml volumes and the aliquots stored at -20°C. Embryos were cultured in the desired concentration of cadmium for specified times and harvested. Control embryos incubated without cadmium were harvested at the same time for enzyme assay.

### 4.2.3 HPRT and APRT microassay

Both injected and control uninjected embryos were harvested at the 2-cell stage, washed in PB1.PVP and transferred in 5 $\mu$l of the

**Figure 4.1** DNA contructions for injection into eggs

**(A)** pDWM1, **(B)** pMT142 and **(C)** pMT-HPRT are reproduced from chapter 3, figure 3.1. All these three (A,B,C) constructs are used in the experiments reported in this chapter.

**(D)** pMT-antiHPRT, the mouse HPRT minigene driven by the metallothionein promoter in the antisense orientation.

**(E)** pMT-antiexon-1HPRT, the cDNA fragment of exon-1 of the HPRT gene cloned next to the metallothionein promoter in the antisense orientation.

Restriction sites: B, <u>Bam</u> H1; Bg, <u>Bgl</u> II; H <u>Hind</u> III; P, <u>Pst</u> I; Pv, <u>Pvu</u> II; R, <u>Eco</u> RI; S, <u>Sal</u> I; X, <u>Xho</u> I. Restriction sites with an asterisk denote artificial sites introduced during the cloning process (for details see Materials and Methods).

# FIGURE 4.1
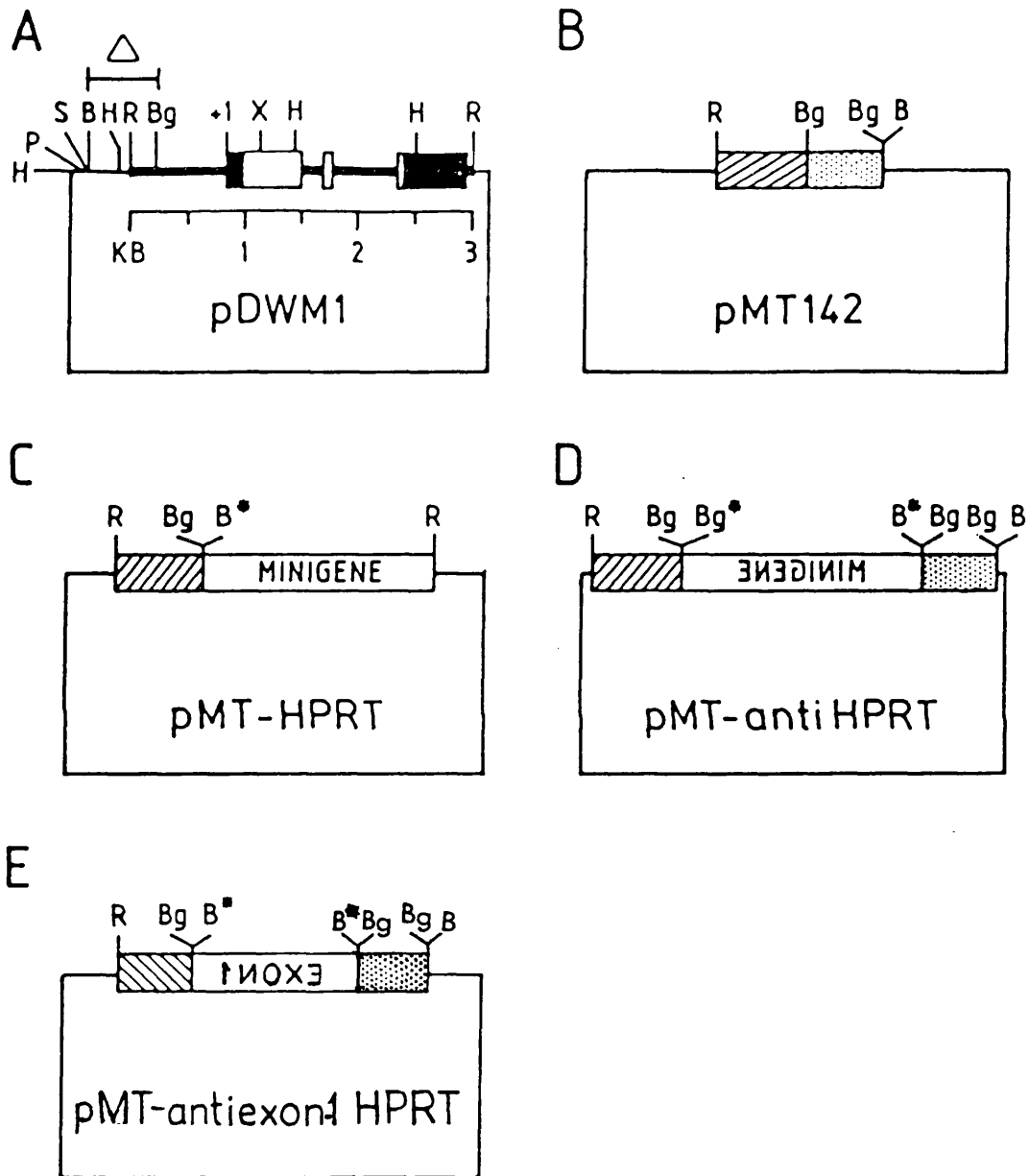
same medium to 10 μl Drummond microcaps. The ends of the microcaps were sealed by melting the glass in a flame and the samples stored at -70°C. Extracts were prepared by freeze-thawing three times and the supernatants assayed for HPRT and APRT as described in chapter 1.2.7. The specific activity of $^3$H-hypoxanthine was 670 or 1180 Ci/M and of $^{14}$C-adenine, 235 Ci/M. The reactions were incubated for 3 hours at 37°C.

## 4.2.4 Plasmid constructions

The HPRT minigene (pDWM1 Δ-638) used in these experiments was derived from the basic construct (pDWM1; chapter 3.2.1, also see Melton, 1986) by deleting material between the Bam HI and Bgl II restriction sites (figure 4.1A). The expression vector, pMT142, was kindly provided by R.Palmiter. The construction of pMT-HPRT (figure 4.1C) plasmid has been explained in chapter 3.2.1. Plasmid pMT-antiHPRT (Figure 4.1D) was constructed by deleting the 5' end of the HPRT minigene to nucleotide position -3, addition of a Bgl II linker to the 3' end, and cloning in the antisense orientation into the Bgl II site of pMT142, thus bringing it under the control of the metallothionein promoter. pMT-antiexon-1HPRT (figure 4.1E) was constructed by cloning the HPRT cDNA fragment (residues 11 to 132; Melton, 1984) into pUC8 as follows: the Eco RI site at the 3' end was cut, the cut converted to a blunt end and a Bgl II linker added. The exon-1 fragment was then released with Bgl II and Bam HI and cloned into the Bgl II site of pMT142, thus bringing it under the control of the metallothionein promoter, as with pMT-antiHPRT.

## 4.2.5 Preparation of DNA for producing transgenic mice

### I Growing of plasmid DNA (pMT-antiHPRT)

For microinjection experiments to produce antisense transgenic mice, the plasmid DNA (pMT-antiHPRT) was prepared in large amount and the vector sequences removed.

### (i) Transformation of bacteria

A single colony of E.coli, JM109, was picked into 5 ml of $\psi$ broth (2% Difco Bacto Tryptone, 0.5% Difco Bacto yeast extract, 0.4% $MgSO_4$, 10 mM KCl, pH 7.6). After overnight culture with aeration at 37°C, 1 ml was added to 100 ml of prewarmed $\psi$ broth and further cultured for 3-4 hours (shaking) until the $OD_{550}$ was 0.48. The cells were chilled on ice for 10 minutes, transferred to two 50 ml Falcon tubes and centrifuged at 2,500 rpm for 5 minutes at 4°C. The pellets were resuspended in 15 ml of ice-cold TfB I solution (100 mM rubidium chloride, 50 $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% glycerol), kept at 4°C for 30 minutes, centrifuged again, and resuspended in 4 ml of ice-cold TfB II solution (10 mM MOPS, pH 7, 10 mM rubidium chloride, 75 mM $CaCl_2$, 15% glycerol). Aliquots of 0.2 ml of these cells were stored at -70°C.

For transformation, an aliquot of cells was thawed at room temperature until just liquid and held on ice for 5-10 minutes before adding the DNA (pMT-antiHPRT: 100 ng/0.2 ml of cells). DNA and cells were kept on ice for 20 minutes, then heat shocked at 42°C for 90

seconds and replaced on ice for 1-2 minutes. After addition of 4 ml

of $\psi$ broth (at room temperature) the cells were cultured at 37°C for

50-60 minutes. 100 µl of cells were spread on L agar plates with 50

µg/ml ampicillin (the vector carrying the pMT-antiHPRT sequences

confers ampiciliin-resistance to the cells).


## (ii) Preparation of plasmid DNA


A single ampicillin-resistant bacterial colony (transformed with

pMT-antiHPRT plasmid) was picked into 10 ml of L broth (1% Bacto

Tryptone, 0.5% Bacto yeast extract, 1% NaCl). After overnight culture

at 37°C, 1 ml was added to 500 ml of L broth with ampicillin (400 µl

of 100 mg/ml solution) and further cultured overnight at 37°C. The

bacterial cells were harvested by centrifugation at 2,500 rpm for 10

minutes at 4°C. The pellets were washed in 50 ml of ice-cold STE (100

mM NaCl, 10 mM Tris, pH 8.0), resuspended in 8 ml of solution I (50 mM

glucose, 25 mM Tris, pH 8, 10 mM EDTA) with lysozyme (5 µg/ml) and the

cells lysed during 5 minutes at room temperature. The lysed bacterial

cells were gently mixed with solution II (0.2N NaOH, 1% SDS) and kept

on ice for 10 minutes. 12 ml of ice-cold potassium acetate (5 M, pH

4.8) was added to the tube, the contents mixed thoroughly by inverting

the tube sharply several times, and replaced on ice for a futher 10

minutes. The bacterial cell DNA and bacterial debris were pelleted by

centrifugation at 20,000 rpm for 20 minutes at 4°C. The supernatant

containing the plasmid (pMT-antiHPRT) was transferred to fresh corex

tubes, 0.6 ml of isopropanol added, the contents were mixed well and

left to stand at room temperature for 15 minutes.

**Figure 4.2A.** The pMT-antiHPRT construct used for microinjection to produce transgenic mice. The 4.54 kb pMT-antisense HPRT insert was isolated by digesting the pMT-antiHPRT construct (figure 4.1D) with Pst I and Sal I. The thin lines, vector sequences; shaded box, mouse metallothionein promoter; stippled box, the 3' end of the human growth gene.

Restriction sites: B, Bam H1; Bg, Bgl II; P, Pst I; R, Eco RI; S, Sal I. Restriction sites with an asterisk denote artificial sites introduced during the cloning process. The length of DNA segments are given in base pairs.


**Figure 4.2B.** Ethedium bromide stained agarose gel showing isolation of linear fragment containing pMT-antiHPRT sequences.

Lane 1 = λ DNA digested with Hind III (marker DNA)

Lane 2 = pMT-antiHPRT uncut plasmid DNA

Lane 3 = pMT-antiHPRT DNA linearised with Sal I

Lane 4 = pMT-antiHPRT DNA linearised with Pst I

Lane 5 = pMT-antiHPRT DNA digested with Sal I and Pst I, thus isolating the antisense HPRT fragment (4.54kb) from the vector sequences (3.09 kb).

# FIGURE 4.2

**A**
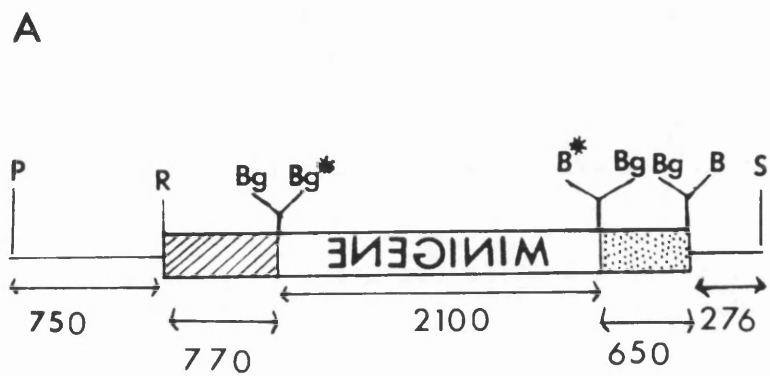


**B**
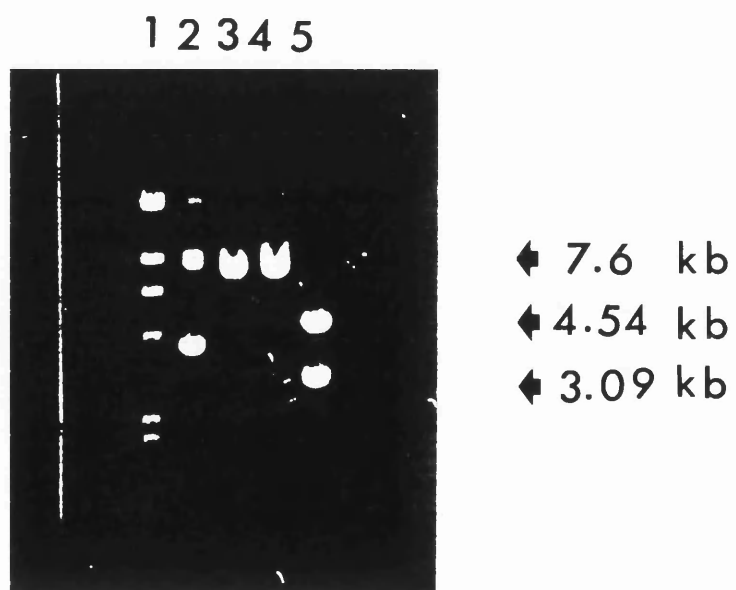
The precipitated plasmid DNA was recovered by centrifugation at 12,000g for 30 minutes at room temperature. The DNA pellet was washed in 70% ethanol, air dried and dissolved in 10 ml TE.

The plasmid DNA was further purified by centrifugation to equilibrium through a caesium chloride gradient containing ethidium bromide (10 mg/ml; Maniatis et al, 1982). The plasmid DNA fraction removed from the gradient was then extracted with equal volumes of butanol saturated with distilled water until the ethidium bromide was completely removed. The DNA solution was dialysed against TE at 4°C overnight. The concentration of plasmid DNA was determined by measurement of OD at 260 nM. The preparation was stored at -20°C.

The cloned pMT-antiHPRT plasmid was linearised and the vector sequences were removed by digestion with Pst I ans Sal I (see figure 4.2).

Digested plasmid DNA was electrophoresed in an ultra pure agarose (BRL) gel, the desired fragment (4.54 kb band) cut out and incubated for 5 minutes at 45-55°C to melt the agarose. The DNA fragment (pMT-antiHPRT insert) was isolated by binding of DNA to powdered glass using 'gene clean' (Bio 101 Inco.) kit. The volume was increased $2\frac{1}{2}$ times by addition of NaI solution and glass mix (5 µl for 5 µg of DNA, provided with the kit) and the mixture placed on ice for 5 minutes. The tubes were centrifuged for 10 seconds and the pellet washed three times with 0.5-1 ml of NEW wash solution (provided with the kit). After the third wash, the pellet was centrifuged again and all the liquid removed. The pellet was resuspended in TE, incubated for 2-3

**Figure 4.3** Embryo transfer pipette.  M, M2 medium; E, embryos; B, air bubbles.

# FIGURE 4.3
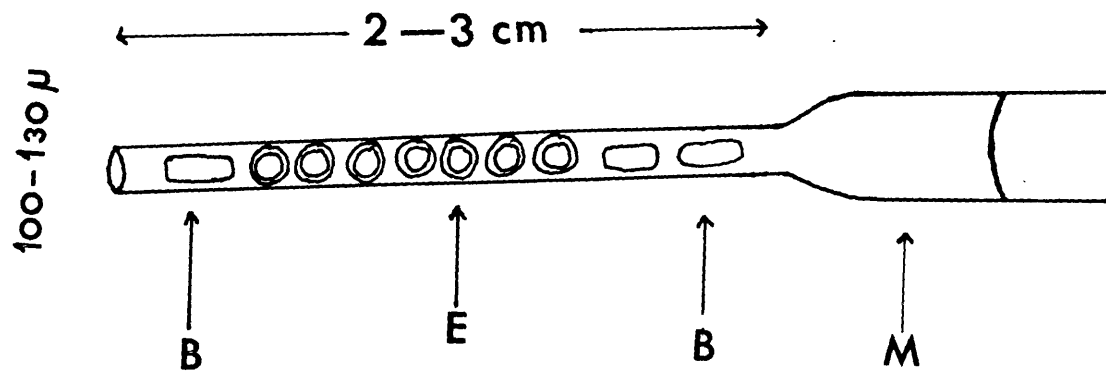
minutes at 45-55°C and spun for 30 seconds. The supernatant was removed from the pellet and kept seperately. The pellet was again washed with TE, the elutes pooled (DNA) and stored at -20°C.

## (iii) Microinjection

Microinjection procedures of DNA into the male pronucleus of fertilised eggs are given in chapter 3.2.8. The fertilised 1-cell eggs were injected with 2 pl of (2 µg/ml) pMT-antiHPRT frament (see figure 4.2) and cultured overnighgt to the 2-cell stage.

## 4.2.6 Embryo transfer

Six week or older F1 (CBA x C57BL) recipient females were mated with vasectomized males the evening before the transfer. The recipient female mouse was anaesthesized by intraperitoneal injection of 0.01 ml of anaesthethic (Hypnorm/Hypnovel in the ratio of 1:2:1 of Hypnorm:water:Hypnovel) per gram of body weight.

A typical embryo transfer pipette is shown in figure 4.3. The pipette was first filled with M2 medium to just past the shoulder, followed by a few air bubbles. Then the embryos (10-15) were picked-up in a minimal volume of medium with a small air bubble followed by a short column of medium. The pipette loaded with embryos was kept undisturbed until it was time to place the embryos in the oviduct.

All the instruments were sterilized by 70% ethanol. After wiping the mouse's shaved back with 70% ethanol, a small transverse incision

was made in the skin at the level of the bottom rib.  Embryos were transferred just to one side oviduct, then to the other, as follows. The fat pad over the ovary, which was visible through the body wall, was located through the incision pulled to one side.  The body wall was picked up with the blunt forceps and a small incision was made just over the ovary.  With the blunt forceps, the fat pad was pulled out together with the attached ovary, oviduct and uterus.  The fat pad was weighed with a metal clip and placed on the middle of the back of the mouse so that the oviduct and ovary remained outside the body wall.  The whole operation was carried out on the stage of the dissecting microscope.

The opening of the oviduct underneath the bursa was gently located and the mouse was arranged so that the pipette could enter the infundibulum at a convinient angle.  Using two pairs of Watchmaker's forceps, a small hole was made in the bursa over the infundibulum. Care was taken not to tear through any large blood vessels.  An edge of the infundibulum was picked up with one pair of forceps and the pipette containig the embryos was inserted down the opening to the ampulla.  The contents of the pipette were discharged until the air bubbles at the end of the pipette had entered the oviduct.  With the help of blunt forceps the fat pad, uterus, oviduct and ovary, were eased back inside the body wall.  The same procedure was repeated on the right side of the mouse.  The body wall was sewn up with one or two stiches and the skin was closed up with wound clips.  The operated mouse was kept warm under a lamp until the anaesthesia had worn off. The recipient females were transferred to the mouse room and their pregnancies and numbers of offspring recorded.

## 4.2.7 Screening for transgenic mice

## I Preparation of DNA from tail tip from progeny resulting from the injected eggs

Directly after ear marking the mouse, the terminal third of the tail was removed with a sharp scissors. The tail tissues were cut into small slices using scissors and the slices were dropped into 2 ml of phosphate buffered saline (PBS) in a polypropyline centrifuge tube on ice. The tail tissues were homogenized for 1-2 minutes. The homogenizer was washed thoroughly between samples. The tubes were spun at 1000-2000 rpm in a bench top centrifuge for 10 minutes. The supernatants were removed carefully and the pellet resuspended in 1 ml of buffer A (75 mM NaCl, 25 mM EDTA, pH 8.0) plus 1 ml of buffer B (10 mM Tris HCl, pH 8.0, 10 mM EDTA, 1% SDS, 400 μg/ml proteinase K). The lysate was incubated at 50°C overnight. Phenol extraction was carried out twice, sodium acetate (pH 5.5) was added to the aqueous phase to a final concentration of 0.2 M and the DNA precipitated by addtion of two volumes of absolute ethanol. The precipitated DNA was retreived with a disposable glass spatula, washed through 70% ethanol, air dried and dissolved in TE buffer. The concentration of DNA was determined by measurement of OD and the DNAs stored at -20°C.

## II Analysis of DNA

## Dot blot analysis

DNA samples (1 µg in 100 µl 2xSSC) were denatured by boiling at 95°C for 5 minutes and chilled on ice for 10 minutes. The DNA samples were then spotted on pre-wetted Nytran (Schleicher & Schuell) filter using dot blot apparatus (Bethesda Research Laboratories). The membrane was air dried, washed briefly in 2xSSC and baked for 1 hour at 80°C. For hybridization, pBR322 was nick translated with $^{32}$P-dCTP using a nick translation kit (Amersham). Hybridization was carried out at 65°C overnight and the filter was exposed to X-ray film.

**Figure 4.4** Schematic diagram of steps involved in the experimental

procedure.  See text for detail.

Figure 4.4

pDWM1 *or* pMT–HPRT
(± pMT–antiHPRT *or*
pMT–antiexon–1HPRT)

DNA

culture (± cadmium)

single embryo
extract

Assay HPRT and APRT

reaction mix
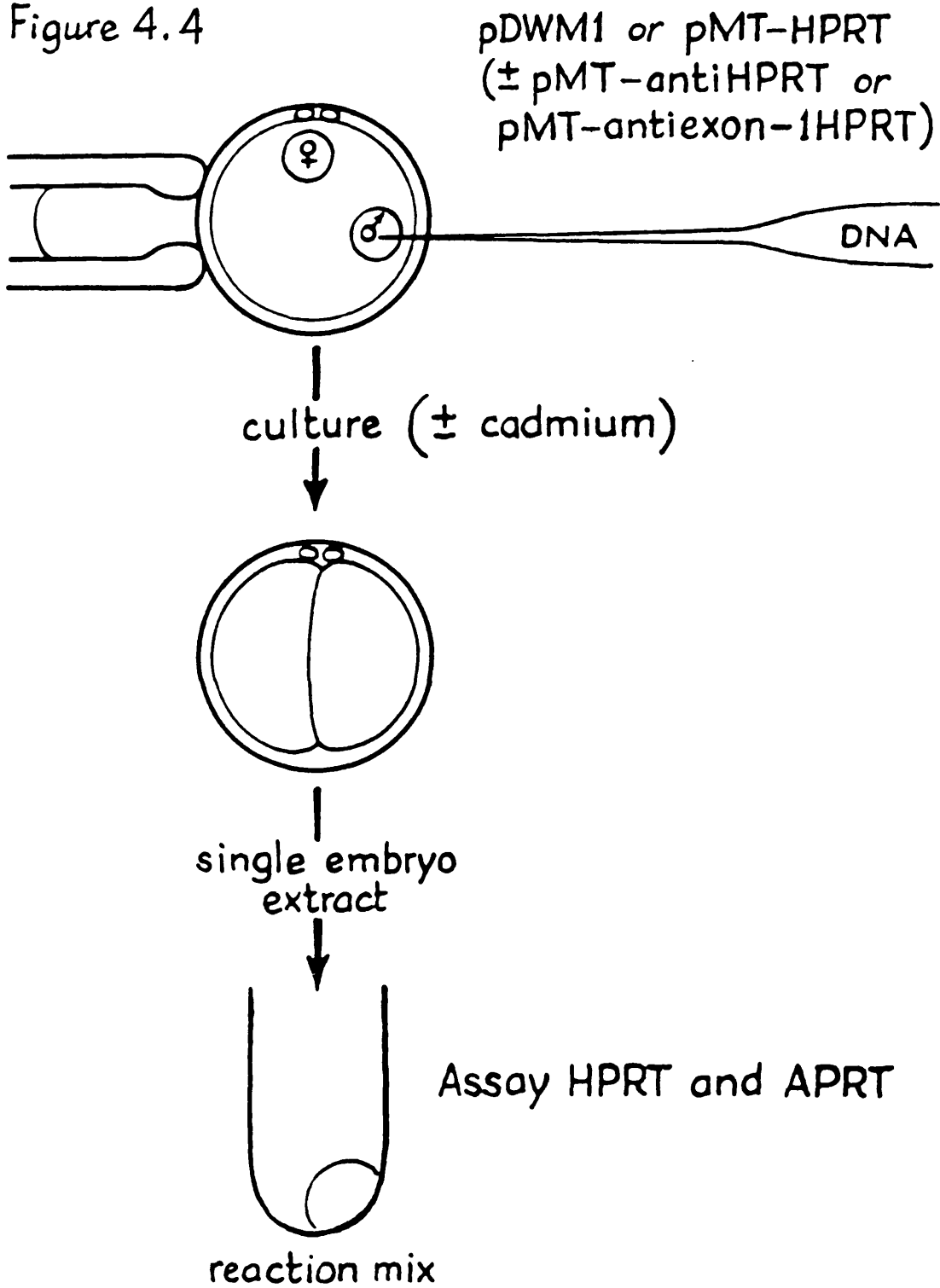
## 4.3 RESULTS

### 4.3.1 Induction of injected HPRT activity by cadmium

The antisense HPRT construct was prepared by cloning the HPRT minigene in the antisense orientation with respect to the inducible mouse MT-I promoter (figure 4.1D). Similarly, another antisense construct was prepared by cloning the exon-1 fragment of the HPRT cDNA clone onto the MT-I promoter in the antisense direction (figure 4.1E). Further constructs used are mouse HPRT minigene with its own promoter (pDWM1Δ-638), HPRT minigene driven by MT-I promoter (pMT-HPRT), the expression vector (pMT142) and pMT-TK (TK gene driven by the MT-I promoter).

To demonstrate HPRT gene activity from the different DNA constructs in mouse embryos, the experiment was carried out as shown in figure 4.4. The male pronuclei of fertilized F1 (CBA X C57 BL) mouse eggs were injected with approximately 2 pl of supercoiled plasmids and cultured in the presence or absence of cadmium. The injected eggs were cultured and collected at the 2-cell stage. Single embryo extracts were added to a reaction mixture to assay for HPRT and APRT activities as described in chapter 3.2.7.

Preliminary experiments were carried out to determine the toxicity of various concentrations of cadmium to morphological development of preimplantation embryos. Table 4.1 shows that concentrations of cadmium above 10 $\mu$M were completely inhibitory to further development. Concentrations in the range of 3-10 $\mu$M allowed

Table 4.1  Development of fertilized eggs in medium with cadmium

| Cadmium concentration (µMolar) | Embryonic development to | | |
|---|---|---|---|
| | 2-cell | 8-cell | blastocyst |
| 20 | - | | |
| 10-3 | + | - | |
| 1 | + | + | + |
| 0 | + | + | + |

.

Table 4.2 Induction of increased HPRT activity by 50 μM cadmium in embryos injected with pMT-HPRT

| Experimental Condition | No of embryos[+] | HPRT* | APRT* | HPRT:APRT |
|---|---|---|---|---|
| A - Injected | 11 | 456 ± 188 | 171 ± 12 | 2.50 ± 0.92 |
| B - Injected (+ Cadmium) | 14 | 1144 ± 309 | 153 ± 24 | 7.29 ± 1.60 |
| C - Uninjected Control | 17 | 179 ± 17 | 195 ± 19 | 0.99 ± 0.15 |
| D - Uninjected Control (+ Cadmium) | 20 | 191 ± 13 | 152 ± 7 | 1.34 ± 0.11 |

*Both HPRT and APRT enzymes are expressed in femtomoles/hour/2-cell embryo

A - Injected embryos cultured continuously for 22 hours without cadmium
B - Injected embryos cultured 15 hours without cadmium followed by 7 hours with cadmium
C - Uninjected embryos cultured continuously for 22 hours without cadmium
D - Uninjected embryos cultured 15 hours without cadmium followed by 7 hours with cadmium
[+]Injected and uninjected control embryos were collected and assayed either singly or in the batches of 2-3 embryos per sample
Replicate experiments gave similar results (data not shown)

Table 4.3  Induction by 1 μM cadmium of increased HPRT activity in embryos injected with pMT-HPRT

| Experimental Condition | No of embryos[+] | HPRT* | APRT* | HPRT:APRT |
|---|---|---|---|---|
| A - Injected | 9 | 423 ± 124 | 103 ± 32 | 2.96 ± 0.69 |
| B - Injected (+ Cadmium) | 10 | 1601 ± 656 | 119 ± 10 | 13.29 ± 6.00 |
| C - Uninjected Control | 10 | 188 ± 11 | 141 ± 9 | 1.34 ± 0.06 |
| D - Uninjected Control (+ Cadmium) | 18 | 197 ± 12 | 146 ± 9 | 1.38 ± 0.15 |

*Both HPRT and APRT enzymes are expressed in femtomoles/hour/2-cell embryo
A - Injected embryos cultured continuously for 22 hours without cadmium
B - Injected embryos cultured continuously for 22 hours in 1 μM cadmium
C - Uninjected embryos cultured continuously for 22 hours without cadmium
D - Uninjected embryos cultured continuously for 22 hours in 1 μM cadmium
[+]The injected and uninjected embryos were collected and assayed either singly or in the batches of 2-3 embryos per sample
Replicate experiments gave similar results (data not shown)

cleavage development to the 2-cell stage and concentrations of 1 µM, or less, allowed apparently normal development. In a previous work, Brinster et al (1982) reported induction of the TK gene fused with mouse MT-I promoter following injection into fertilized mouse eggs and culture of the embryos for 22 hours in 50 µM of cadmium. Since, in our hands, 50 µM of cadmium arrested development at the 1-cell stage, the injected embryos were cultured in medium without cadmium for 15 hours followed by pulse-treatment with 50 µM cadmium added to the 2-cell embryos for a further 7 hours (table 4.2). Injected embryos were also treated continuously for 22 hours in 1 µM cadmium (table 4.3). The results in tables 4.2 and 4.3 show a marked and significant increase in HPRT activity in embryos resulting from the injected eggs following culture for 7 hours at the 2-cell stage in 50 µM cadmium as well as in the continued presence of 1 µM cadmium from the 1-cell stage, compared to injected embryos cultured in M16 medium alone. The endogenous HPRT and APRT enzyme activities were not affected by the cadmium treatment.

## 4.3.2 Negation of gene activity by antisense DNA

To investigate the effect of simultaneous injection of antisense HPRT DNA on the enzyme activity expressed from the exogenous HPRT minigene DNA, the two constructs were injected together with a five-fold molar excess of antisense HPRT DNA (pMT-antiHPRT). In one series of control injected eggs, the HPRT minigene was injected alone. In another series of control injected eggs a five-fold molar excess of plasmid DNA other than the antisense plasmid (e.g. pMT-TK, Brinster et al, 1982 or pMT142, figure 4.1B), in place of antisense DNA, was

Table 4.4 Inhibition of transient expression of HPRT activity from the injected HPRT minigene by simultaneous injection of antisense pMT-HPRT DNA

| Exp number | Injected plasmid[1] | No. of embryos[2] | HPRT[3] | APRT[3] | HPRT:APRT |
|---|---|---|---|---|---|
| I | 2 pMT-HPRT +pMT-TK | 11 | 266 + 55 | 110 + 18 | 2.43 + 0.23 |
| | 3 pMT-HPRT +pMT-antiHPRT | 8 | 133 + 41 | 110 + 28 | 1.29 + 0.17 |
| | 4 Uninjected control | 18 | 189 + 14 | 163 + 20 | 1.24 + 0.14 |
| II | 1 pDWM1Δ-638 | 4 | 257 + 59 | 215 + 14 | 1.23 + 0.20 |
| | 2 pDWM1Δ-638 +pMT142 | 10 | 210 + 48 | 175 + 13 | 1.25 + 0.39 |
| | 3 pDWM1Δ-638 +pMT-antiHPRT | 5 | 123 + 14 | 187 + 56 | 0.79 + 0.15 |
| | 4 Uninjected control | 18 | 101 + 5 | 156 + 12 | 0.67 + 0.06 |
| III | 1 pDWM1Δ-638 | 10 | 133 + 19 | 170 + 11 | 0.83 +0.16 |
| | 2 pDWM1Δ-638 +pMT142 | 4 | 162 + 22 | 172 + 7 | 0.95 + 0.17 |
| | 3 pDWM1Δ-638 +pMT-antiHPRT | 4 | 56 + 14 | 146 + 30 | 0.41 + 0.14 |
| | 4 Uninjected control | 10 | 78 + 5 | 136 + 12 | 0.58 + 0.09 |

[1]Fertilized eggs were injected with pMT-HPRT or pDWM1Δ-638 together with a fivefold excess of either pMT-antiHPRT or, in controls, pMT-TK or pMT-142. (pMT-TK was kindly provided by R.L.Brinster).
[2]Injected eggs were collected after 22 hours and assayed singly.
[3]HPRT and APRT enzymes are expressed in fentomoles/hour/embryo.
Replicate experiments were done three times and gave similar results (data not shown).

injected together with HPRT minigene DNA. The resulting HPRT activity was again assayed at the 2-cell stage. The results are shown in table 4.4.

In embryos injected with the HPRT minigene (line 1, experiments II and III, table 4.4), the usual two-fold increase in HPRT activity over the uninjected control is observed. Co-injection of a five-fold excess of pMT-TK or pMT142 DNA did not have any deleterious effect on cleavage at the 2-cell stage or on the increased expression of HPRT activity from the injected minigene (line 2, experiments I, II and III, table 4.4). However, the co-injection of a five fold excess of antisense DNA reduced HPRT expression to the level found in uninjected controls (line 3, experiments I, II and III, table 4.4). The APRT activity showed similar values in all groups of injected and uninjected control embryos showing that APRT activity is not affected by the injection of unrelated DNA (table 4.4).

### 4.3.3 Negation by antisense DNA to the exon-I region of HPRT

It is of interest to determine what region or regions of antisense DNA are effective in inhibition of expression and how much of the antisense sequences are required. In preliminary experiments, we tested the antisense fragment of exon-I of HPRT gene. The 121 bp (residues 11 to 132, Melton, 1984) of exon-I fragment of HPRT gene, fused to the MT-I promoter (pMT-antiexon-1HPRT), was injected into mouse eggs, simultaneously with HPRT minigene, in the ratio of 5:1, respectively. The results (table 4.5) show similar reduced HPRT expression as was observed with pMT-antiHPRT (table 4.4). The control

Table 4.5  Inhibition of transient expression of HPRT activity from the injected HPRT minigene by simultaneous injection of antisense Exon-1HPRT DNA

| Injected plasmid[1] | No. of embryos[2] | HPRT[3] | APRT[3] | HPRT:APRT |
|---|---|---|---|---|
| pDWM1Δ-638 +pMT142 | 11 | 168 ± 38 | 116 ± 10 | 1.68 ± 0.46 |
| pDWM1Δ-638 +pMT-antiHPRT | 7 | 82 ± 11 | 105 ± 8 | 0.78 ± 0.09 |
| pDWM1Δ-638 +pMT-antiexon-1HPRT | 7 | 79 ± 21 | 98 ± 8 | 0.74 ± 0.13 |
| Uninjected control | 8 | 89 ± 9 | 129 ± 12 | 0.74 ± 0.10 |

[1]Fertilized eggs were injected with pDWM1Δ-638 together with a fivefold excess of either pMT-antiHPRT or pMT-antiexon-1HPRT, and, in controls, pMT-142.
[2]Injected eggs were collected after 22 hours and assayed singly.
[3]HPRT and APRT enzymes are expressed in femtomoles/hour/embryo.
Replicate experiments gave similar results.

embryos injected with HPRT minigene together with a five-fold excess of other DNA (pMT142) again did not show repression of the increased expression of HPRT activity from the injected minigene, nor was there any deleterious effect of the additional DNA injected on cleavage to the 2-cell stage. The endogenous APRT enzyme activities were similar in control and injected embryos.

## 4.3.4  Effect of injection of antisense constructs into cytoplasm

Several workers have shown inhibition of exogenous and endogenous gene expression by direct microinjection of antisense RNA into the egg cytoplasm (see Introduction). In the previous experiments, a plasmid designed to produce antisense RNA repressed HPRT activity when injected into the nucleus (tables 4.4 and 4.5). An experiment was designed to test whether endogenous maternal HPRT mRNA (up to the 2-cell stage), as well as embryo-coded transcripts (from the 4-cell stage), can be inhibited by direct microinjection of antisense DNA into the egg cytoploasm. Cytoplasmic injection is comparatively easier than nuclear injection and this was a simpler experiment to perform (though transcription would not be expected in the cytoplasm).

Fertilized 1-cell eggs were injected with either 5 µg/ml, or a much higher concentration (50 µg/ml), of pMT-antiHPRT DNA into the egg cytoplasm and cultured up to the morula stage. The injected and the control uninjected embryos were collected at 2-cell, 5- to 8-cell and morula stages after 1, 2 and 3 days of culture, respectively. It was found that the injected embryos showed no reduction in endogenous HPRT activity at the 2-cell, 5- to 8-cell or morula stages compared to the

uninjected control embryos (data not shown). This result shows that antisense HPRT DNA does not inhibit endogenous HPRT gene expression, even from the maternal mRNA at the 2-cell stage, when injected into the egg cytoplasm.

### 4.3.5 Inducible antisense constructs

Since the inducibility of pMT-HPRT was shown by increased HPRT expression in mouse embryos (see tables 4.2 and 4.3), an experiment was designed to test the negation of gene activity by inducible HPRT antisense constructs. This approach was taken to see if a reduced proportion of antisense to sense molecules injected into the embryos would be effective in inhibition of gene activity. Such an approach is important in considering whether antisense transgenic mice can be subjected to conditional gene expression using inducible promoters.

In the previous antisense experiments a five-fold excess of antisense DNA to sense DNA was used. In this experiment, the fertilized eggs were injected with equal amounts of antisense (pMT-antiHPRT) and sense (pDWM1Δ-638) DNAs (ratio 1:1) and were cultured in medium without cadmium for 15 hours followed by addition of 50 μM cadmium to the resulting 2-cell embryos for a further 7 hours. One series of eggs were similarly injected with equal amounts of antisense and sense DNA (ratio 1:1) and cultured to the 2-cell stage in the absence of cadmium. Two other groups of embryos were included in the experiment; in one group the eggs were injected with sense plus antisense in five-fold excess, in another control group, with sense plus control DNA plasmid (pMT142, in five-fold excess). These groups

Table 4.6   Cadmium-induced inhibition of HPRT expression by pMT-antiHPRT

| | Injected plasmid[1] | Ratio of plasmids | No. of embryos[2] | HPRT[3] | APRT[3] | HPRT:APRT |
|---|---|---|---|---|---|---|
| 1 | pDWM1Δ-638 +pMT142 | 1:5 | 6 | 187 ± 44 | 165 ± 21 | 1.07 ± 0.16 |
| 2 | pDWM1Δ-638 +pMT-antiHPRT | 1:5 | 10 | 80 ± 8 | 153 ± 2 | 0.54 ± 0.05 |
| 3 | pDWM1Δ-638 +pMT-antiHPRT | 1:1 | 7 | 149 ± 17 | 166 ± 10 | 0.90 ± 0.12 |
| 4 | pDWM1Δ-638 +pMT-antiHPRT | 1:1 | 4 | 80 ± 15 | 133 ± 5 | 0.61 ± 0.12 |
| 5 | Uninjected control | - | 8 | 76 ± 3 | 175 ± 13 | 0.45 ± 0.03 |

[1]Fertilized eggs were injected with pDWM1Δ-638 together with a fivefold excess or equal amount of pMT-antiHPRT or in controls, five fold excess of pMT142. For induction in line 4 the injected eggs were cultured 15 hours without cadmium followed by 7 hours in 50 μM cadmium.
[2]Injected and uninjected eggs were collected after 22 hours and assayed singly.
[3]HPRT and APRT enzymes are expressed in femtomoles/hour/embryo.

Table 4.7  Effect of different ratios of inducible antisense to sense DNA in injected mouse embryo

| Injected plasmid[1] | Ratio of plasmids | No. of embryos[2] | HPRT[3] | APRT[3] | HPRT:APRT |
|---|---|---|---|---|---|
| pDWM1Δ-638  pMT-antiHPRT | 1:0.1 | 6 | 230 ± 41 | 213 ± 19 | 1.07 ± 0.16 |
| pDWM1Δ-638 +pMT-antiHPRT | 1:0.5 | 11 | 175 ± 34 | 185 ± 16 | 1.04 ± 0.24 |
| pDWM1Δ-638 +pMT-antiHPRT | 1:1 | 10 | 88 ± 7 | 230 ± 16 | 0.39 ± 0.03 |
| pDWM1Δ-638 +pMT-antiHPRT | 1:1 | 8 | 160 ± 29 | 192 ± 26 | 0.77 ± 0.13 |
| Uninjected control | - | 9 | 86 ± 10 | 256 ± 21 | 0.35 ± 0.04 |

[1]Fertilized eggs were injected with pDWM1Δ-638 together with pMT-antiHPRT in the ratio of 1:0.1, 1:0.5 and 1:1 as shown. The induced injected eggs were cultured 15 hours without cadmium followed by 7 hours in 50 µM cadmium.
[2]Injected and uninjected eggs were collected after 22 hours and assayed singly.
[3]HPRT and APRT enzymes are expressed in femtomoles/hour/embryo.
The samples were blind coded for the assay. Replicate experiments gave similar results (data not shown).

were also cultured to the 2-cell stage in M16 medium without cadmium.
The results are shown in tables 4.6 and 4.7. As expected the embryos
injected with 1:5 sense to antisense DNA showed HPRT activity reduced
to the basal level, whereas, the embryos injected with 1:5 sense to
control DNA (pMT142) showed a two-fold increase in HPRT activity
compared with the control uninjected embryos. The embryos injected
with equal amounts (1:1) of sense and antisense DNA and cultured in
the presence of cadmium showed efficient antisense negation of
exogenous HPRT expression. No negation was obtained when the co-
injected embryos were cultured in the absence of cadmium. This result
is interesting because it would provide a convenient way to manipulate
the amounts of antisense RNA synthesized in cells using an inducible
promoter. In this way, a conditional expression of antisense
inhibition could operate under the control of the inducing agent.

It was of interest to see whether ratios of antisense to sense
DNA could be further reduced from 1:1 to 0.5:1 and 0.1:1 and still
show inducible inhibition of gene expression. The co-injected embryos
were pulse treated with 50 μM cadmium at the 2-cell stage as described
above. The results (table 4.7) show no reduction of increased HPRT
activity with antisense to sense proportion below a 1:1 ratio.

### 4.3.6 The production of antisense transgenic mice

Following the successful negation of gene expression by the
inducible anitisense HPRT construct in early mouse embryos (see
section 4.3.5 and table 4.6), an attempt was made (using the same
antisense construct, pMT-antiHPRT but with vector sequences removed)

to produce transgenic mice in order to create an inducible system in later stages of development and in adult mice.

The cloned DNA (pMT-antiHPRT) used for microinjection to create transgenic mice was prepared in a linearised form with as much vector sequence removed as possible by digesting with Pst I and Sal I (see figure 4.3). The linearised DNA has a five-fold higher integration frequency compared with circular molecules (Brinster et al, 1985). Although the removal of vector sequences does not alter DNA integration frequency, their presence may have a profound effect on subsequent expression of the transgene with a drop in expression of up to 1000-fold (Chada et al, 1985).

For microinjection purposes, DNA purity is of utmost importance. The DNA sample must be free of organic solvent contaminants which have a severe detrimental effect on the developing embryo. Therefore the DNA was isolated and purified without the use of phenol, chloroform or ether (see 4.2.5 part I)

For the microinjection experiments (to produce transgenic mice), a DNA concentration of 2 µg/ml was used. This corresponds to ~400 copies of DNA of 5 kb fragment in 2 pl of DNA, the volume that can be injected into the pronucleus. Brinster et al (1985) achieved optimal DNA integration efficiency (20-40%) with concentrations of linear DNA of 1 µg/ml or higher. However, at higher DNA concentrations (>10 µg/ml) embryo survival decreased significantly.

**Figure 4.5** Dot blot analysis of mouse tail DNA indicating six

positive signals for DNA of transgenic mice carrying the pMT-antiHPRT

construct. The large spot is control DNA (pBR322 containing the

adenosine deaminase gene) which was overloaded in this experiment.

FIGURE 4.5
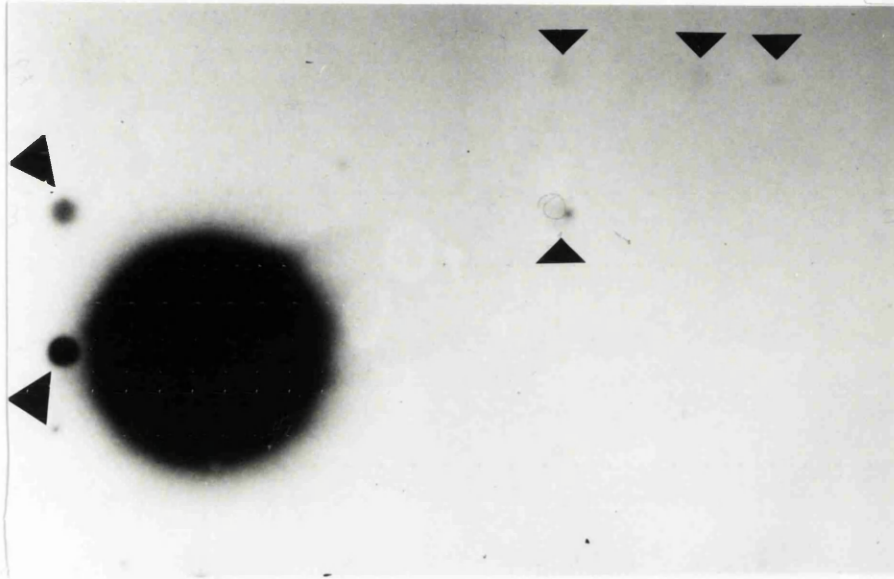
The fertilized eggs which survived microinjection, were cultured overnight and the resultant 2-cell embryos transferred into the ampullae of day 1 pseudopregnant recipients. This procedure is called an oviduct transfer. Approximately 10-30% of the microinjected eggs transferred into the oviduct will develop to term. To avoid very small litters, which may not be cared for by the foster mother, 20-30 microinjected eggs were transferred into each pseudopregnant recipient.

Following the transfer of embryos to the oviduct, pregnancies were monitored from time to time. In our mouse colony healthy young are weaned at 21 days after birth. At this time they are big enough to be checked for presence of injected DNA sequences by tail analysis. Tail biopsies and numbering of the animals by ear punching, were done at the same time.

Transgenic animals resulting from injected embryos were initially identified by dot blot analysis because this method is simpler and quicker compared to Southern blot analysis. However, the additional information provided by the Southern blot e.g estimation of number of integration sites and of copy number, will be required at a later stage.

The dot blot analysis of tail DNA showed that 6 out of 23 live born individuals were positive for the HPRT antisense construct (figure 4.5). Phenotypically all six transgenic mice looked normal. Blood samples were taken from tails of transgenic, control non-transgenic mice (from injected eggs but negative for the antisense

Table 4.8 Analysis of HPRT activity in blood samples of transgenic and control mice

| Mouse type | No of mice* | HPRT& | APRT& | HPRT:APRT |
|---|---|---|---|---|
| A | 1 | 2090 | 14290 | 0.15 |
|   | 2 | 1460 | 6644 | 0.22 |
|   | 3 | 1773 | 15526 | 0.11 |
|   | 4 | 874 | 2573 | 0.34 |
| B | 1 | 903 | 4643 | 0.19 |
|   | 2 | 2666 | 6699 | 0.40 |
|   | 3 | 1598 | 4282 | 0.37 |
| C | 1 | 5350 | 44441 | 0.12 |
|   | 2 | 1871 | 17213 | 0.11 |

A = transgenic mice; B = control non-transgenic mice (negative for the antisense construct); C = control F1 mice of same age as those of groups A and B.
* one sample/mouse.
& HPRT and APRT enzymes are expressed in femtomoles/hour/5 μl of diluted blood sample.
Specific activities of $^3$H-Hypoxanthine and $^{14}$C-Adenine, 1180 Ci/M and 270 Ci/M respectively.

HPRT construct) and control F1 mice, of the same age as the transgenic mice. The blood samples were diluted 1/100 with PB1.PVP and assayed for HPRT activity (see chapter 1.2.7). The results in table 4.8 showed no difference in HPRT activity and HPRT:APRT ratio among these three groups of mice. However, these data are preliminary and more experiments need to be done to extend these results.

## 4.4 DISCUSSION

### 4.4.1 Induction of injected HPRT activity by cadmium

Metallothionein genes are expressed in most animal tissues and cell lines (Mayo & Palmiter, 1981; Durnam & Palmiter, 1981). The synthesis of mouse MT-I mRNA is transcriptionally controlled by heavy metals (Durnam & Palmiter, 1981), glucocorticoid hormones (Mayo & Palmiter, 1981; Hager & Palmiter, 1981) and by a hormone liberated in response to inflammation (Durnam et al, 1984; Searle et al, 1984).

The inducibility of mouse MT-I promoter has been shown by several workers. In addition, direct subcutaneous injections of cadmium into the mouse shows increased MT synthesis (Durnam & Palmiter, 1981). The MT-I promoter has been fused to other gene sequences enabling their increased expression by induction with heavy metals. For example, TK activity was regulated by addition of cadmium to the medium of mouse TK⁻ L cells transfected with a construct of the MT-I promoter fused with the TK gene (Mayo et al, 1982). Induction of transcription using the MT-I promoter has also been shown in embryos; when a recombinant DNA construct consisting of mouse MT-I promoter fused to the structural gene of Herpes Virus TK gene was injected into mouse eggs, a 10-fold increase in TK activity was observed upon cadmium treatment (Brinster et al, 1982).

In addition to metallothionein promoters, other inducible promoters have been reported (Wu, 1984; Goodbourn et al, 1985; Topol et al, 1985; Holt et al, 1986). The mouse MT-I promoter was used in

our experiments because its inducibility in the mouse embryo is established (Brinster et al, 1982). However, in our hands, continuous culture in 50 μM cadmium was found to be toxic to mouse embryo development. Other studies have also shown that higher concentrations of cadmium are embryotoxic in mouse and rabbit embryos. Yu et al (1985) have shown that in vitro exposure of preimplantation mouse embryos to 10 μM cadmium is embryotoxic, leading to necrosis of morulae within 24 hours. A similar result was observed by Andrews et al (1987) where greater than 10 μM cadmium rapidly caused the day-6 blastocyst to collapse and severely reduced the incorporation of [$^{35}$S]-cysteine into protein. In this chapter preliminary experiments were carried out to determine the toxicity of various concentrations of cadmium to ongoing morphological development of preimplantation mouse embryos. It was found that concentrations of cadmium above 10 μM are completely inhibitory to further development, concentrations in the range of 3-10 μM allow one cleavage division, and concentrations of 1 μM, or less, allowed the embryos to reach the blastocyst stage. Therefore, in the experiments reported here, embryos were either pulse-treated with 50 μM cadmium for 7 hours or cultured continuously in 1 μM cadmium.

Embryos which have been injected with pMT-HPRT and induced either by continuous culture in the presence 1 μM cadmium or pulse-treated with 50 μM cadmium show a marked and significant increase in HPRT activity. The fact that treatment with 50 μM cadmium at the 2-cell stage shows induction of HPRT activity in the injected embryos suggests that the inducible HPRT minigene is still present at this time, at least 15 hours after microinjection. Previous experiments

(chapter 3.3.1) showed no further increase in exogenous HPRT activity beyond the 2-cell stage when cultured to the 5-8-cell and morula stages, suggesting degradation or inactivation of injected HPRT minigene DNA. However, the injected plasmid DNAs must remain transcriptionally active for at least 15 hours after injection.

## 4.4.2 Inhibition of gene expression by antisense DNA or RNA

The use of antisense mRNA to block expression of specific gene products offers considerable potential as a means of investigating gene function in vivo. A series of recent studies have shown inhibition of gene expression, presumably by inhibition of translation of specific mRNAs, by direct injection of antisense RNA into the cytoplasm of Xenopus oocytes (Harland & Weintraub, 1985; Melton, 1985; Wormington, 1986). Repression of ribosomal protein, L1, synthesis was achieved within 12 hours after microinjection of antisense RNA and was maintained for 48 hours. Formation of RNA:RNA duplexes in vivo between the endogenous L1 mRNA and injected antisense transcripts was shown by RNase protection assay (Wormington, 1986).

The use of direct microinjection of antisense RNA into the egg cytoplasm to inhibit endogenous gene expression was also extended to mouse embryos (Bevilacqua et al, 1988; Bevilacqua et al, 1989). The biological role of gap junction (GJ) was explored in preimplantation mouse embryos by disrupting GJ synthesis by injecting antisense RNA. When an antisense RNA coding for rat liver GJ protein was injected into the blastomeres of 2- and 4-cell embryos, less than 20% of the embryos compacted. When the blastomeres of 8-cell embryos were

injected with the same antisense RNA, only 5% developed to the blastocyst stage compared to 89% of the uninjected control and 100% of the control embryos injected with β-glucuronidase sense RNA (Bevilacqua et al, 1989).

In Drosophila, introduction of Krüppel antisense RNA into the embryo results in mutant phenotypes (Rosenberg et al, 1985).

Inhibition of gene expression by antisense RNA generated in vivo from DNA vectors has been successfully achieved in a variety of systems. However, interference with gene expression in mammalian eggs by antisense DNA constructs has not been previously reported. The results presented here (table 4.4) suggest that a plasmid designed to produce antisense RNA may repress the expression of exogenously introduced HPRT genes in mouse embryos. The fact that antisense DNA did not reduce the (basal) level of endogenous HPRT activity, which is attributable at this stage to maternally-inherited enzyme and mRNA (MmRNA) (Harper & Monk, 1983; and see chapter 1.3 of this thesis), suggest that the antisense DNA exerts its effect only within the nucleus. An attempt to try to inhibit maternal and embryonic HPRT mRNA by direct microinjection of plasmid DNA into the egg cytoplasm did not repress endogenous HPRT activity in the mouse embryo.

In systems other than mammals, antisense DNA (rather than RNA) has resulted in repression of specific gene expression (Izant & Weintraub, 1984; Coleman et al, 1984; Pestka et al, 1984; Kim & Wold, 1985; McGarry & Lindquist, 1986; Holt et al, 1986). A plasmid constructed to generate RNA complementary to the β-galactosidase mRNA

inhibited synthesis of $\beta$-galactosidase by 98% in E.Coli (Pestka et al, 1984). Similarly, McGarry & Linquist (1986) showed inhibition of hsp26 synthesis by introducing antisense hsp26 DNA into a Drosophila tissue culture system. Crowley et al (1985) induced a mutant phenotype in Dictyostelium by antisense DNA. Following transformation with an antisense construct of the discoidin gene, phenocopies of the Dictyostelium discoidin-minus mutant resulted from the repression of three endogenous discoidin genes. These mutants produced by antisense DNA cannot form streams on a plastic surface due to altered cell-substratum interaction (Crowley et al, 1985). Transformants exhibited a greater than 90% reduction in accumulated discoidin mRNA and protein.

### 4.4.3 Mechanism of inhibition of gene expression by antisense RNA or DNA

The mechanism by which antisense RNA, used directly or synthesised from antisense DNA, inhibits expression of a gene has been shown to vary depending on the system studied. In bacteria, it has been found to inhibit translation, presumably by interfering with ribosome binding (Mizuno et al, 1984; Simon & Kleckner, 1983). In eukaryotes, the precise mechanism of inhibition remains unclear but appears to involve the formation of a stable duplex between antisense and sense RNA. This either prevents the processing and transport of mRNA from the nucleus (Kim & Wold, 1985) or ribosome binding and translation of mRNA in the cytoplasm (Melton, 1985), depending on the cellular location of the sense and antisense RNA. In Dictyostelium, a different result was found where the mechanism of inhibition appeared

to be the formation and subsequent degradation of double-stranded RNA in the nucleus (Crowley et al, 1985).

## 4.4.4 Region and extent of antisense sequence required

In the experiments reported in this chapter, inhibition of injected HPRT gene expression was obtained in the early mouse embryo by injection of antisense HPRT exon-1 cDNA fragment fused to the MT-I promoter (pMT-antiexon-1HPRT, figure 4.1E; table 4.5). The pMT-antiexon-1HPRT construct contains 121 bp of exon-1 HPRT cDNA, compared to the much longer HPRT minigene (2.1 kb) cloned to produce pMT-antiHPRT. However, both the constructs inhibited HPRT minigene expression in mouse embryos (tables 4.4 and 4.5). Several studies have shown that an entire antisense RNA is not required to inhibit the translation of the mRNA (see review by Green et al, 1986).

To date, there is no precise information on which regions of an RNA transcript are most susceptible to antisense inhibition. In frog oocytes, $\beta$-globin antisense RNA covering only the 5' untranslated region of the mRNA was as effective as the entire mRNA in blocking translation (Melton, 1985). A similar conclusion was reached for the inhibition of nicotinic acetyl choline receptor (AcChoR) of the electric organ of Torpedo in Xenopus oocytes. Sumikawa and Miledi (1988) suggested that an important antisense region for repressing the translation of the AcChoR's $\alpha$-subunit mRNA is the 5' region of the mRNA, but that it is not necessary to cover the ribosome binding site or the initiation codon. Izant & Weintraub (1985) also found 5' antisense RNA a more efficient inhibitor in cultured cells than

protein coding sequences alone. However, in contrast, Kim and Wold
(1985) observed the same degree of inhibition of Herpes Simplex TK
with antisense constructs complementary to either the 3' or 5' end of
the gene. A similar result was obtained by Wormington (1986) in
Xenopus oocytes. Antisense RNAs complementary to only the 3' position
of L1 mRNA were as effective in repression as RNA spanning the entire
L1 coding region. These results are also supported by those of
Harland and Weintraub (1985) who found that the 3' end of CAT
antisense RNA is able to partially inhibit CAT expression in Xenopus
oocytes. Furthermore, Holt et al (1986) suggested that complete
homology is not required for effective inhibition of gene function by
antisense DNA sequences. The inhibition of c-fos gene function was
observed with an antisense construction containing a fragment of the
human c-fos gene, which had about 80% homology with the corresponding
portion of the mouse c-fos gene. Thus, it appears that the extent and
region of homology required for effective antisense inhibition is
variable and must be tested experimentally.

### 4.4.5  Stability of injected plasmids in the embryo

The stability of injected antisense DNA or RNA may be another
factor which contributes to effective inhibition of target mRNA. The
expression of injected HPRT minigene was monitored throughout
preimplantation development of the mouse embryo (see chapter 3; table
3.1). The embryos injected with HPRT minigene showed two-fold
increase in HPRT activity over the control uninjected embryos at the
2-cell stage. However, the increase in HPRT activity was not observed
after the 2-cell stage; the injected embryos showed similar endogenous

HPRT activities to control embryos at the 5- to 8-cell, morula, and blastocyst stages. The cessation of new enzyme synthesis from the HPRT minigene beyond the 2-cell stage may be due to degradation or inactivation of the injected DNA after the initial active transcription. Alternately, new enzyme activity synthesized from the HPRT minigene may be undetectable above that due to the onset of embryonic HPRT enzyme activity. Increased embryonic HPRT enzyme activity is observed after the 2-cell stage and continues to the blastocyst stage (table 1.3, figure 1.4). However, table 4.6 shows that the inducible antisense DNA is still present at the 2-cell stage. The inhibition of HPRT minigene was still possible in the embryos injected together with inducible antisense DNA and pulse-treated with cadmium after 15 hours of injection. The results suggest that the injected DNA does not get degraded or inactivated for at least 15 hours after injection.

### 4.4.6 The antisense to sense ratio required for inhibition

The ratios of the antisense RNA to sense RNA needed to achieve significant inhibition of the target message in previous reports is variable (Izant & Weintraub, 1984; Melton, 1985; Wormington, 1986). In some of the cases reported (Harland & Weintraub, 1985; Melton, 1985), the excess antisense RNA to sense RNA required was 20- to 50- fold or greater (Wormington, 1986) and even then inhibition was not complete in some cases. When antisense DNA was used in transfection studies, a five-fold excess of antisense over sense could lower the target message up to 5- to 20-fold compared to the control cells (Izant & Weintraub, 1985). In other cases, as much as 100- to 200-

fold excess antisense DNA was microinjected to give significant inhibition in transient message (Izant & Weintraub; 1984, 1985). In the experiments reported here (tables 4.4 and 4.5), a five-fold excess of antisense DNA to sense DNA was used. The five-fold excess of antisense DNA was sufficient to reduce HPRT expression to the level found in uninjected controls.

### 4.4.7 Inducible antisense DNA in developmental studies

For developmental studies, it would be of great advantage if antisense DNA could be designed to inhibit endogenous gene expression in specific tissues and at a specific developmental stage. To achieve a significant reduction in the expression of particular genes, high amounts of RNA may be required (see above). However, a high concentration of injected antisense DNA required to achieve this, might be toxic to embryos (Gurdon & Brown, 1977; Rusconi & Schaffner, 1981) and the available expression plasmids may not produce sufficient amounts of RNA quickly enough to inhibit genes turned on in the rapidly dividing embryo. To overcome such problems, plasmids may be designed to target the site and time at which antisense RNA is supplied by using tissue-specific and inducible promoters to control the expression of antisense RNA.

The glucocorticoid-inducible mouse mammary tumor virus (MMTV) promoter and heat-inducible heat-shock protein promoter have been used to drive antisense gene transcription. McGarry & Lindquist (1986) transformed Drosophila tissue culture cells with a heat-inducible antisense hsp 26 gene and, following heat induction, the heat-shock

inducible protein hsp 26 was inhibited by 89% compared with untransformed cells. The inhibition was specific since other, closely related, heat-shock proteins did not appear to be affected. A similar inhibition of the c-fos gene was observed using a steroid-inducible MMTV promoter in mouse 3T3 cells (Holt et al, 1986). The inducible MMTV promoter was also used to inhibit TK enzyme activity in mammalian TK⁺ cells (Weintraub et al, 1985; Izant and Weintraub, 1985).

The experiments reported in this chapter show the inducibility of pMT-antisense construct by cadmium treatment (table 4.6). Inhibition of exogenous HPRT gene expression was observed with a reduced proportion of antisense DNA upon cadmium induction. The results suggest that it is possible to generate enough antisense RNA to achieve inhibition of a target gene by using as little as a 1:1 ratio of inducible antisense plasmids to sense plasmids.

The results also show that equal gene copies of antisense to sense will not be effective in inhibition of specific gene expression but inhibition of specific gene expression will be achieved when the antisense expression is induced. This result is exciting because it suggests that inhibition of gene expression in mice transgenic for specific antisense gene constructs could be made conditional and be induced at specific times in development.

### 4.4.8 The production of antisense transgenic mice

Transgenic mice have been used to study the molecular basis of tissue specific and stage specific gene expression during mouse

development. Most cloned genes that have been introduced into the mouse germ line have shown appropriate patterns of expression (e.g. Chada et al, 1985, Palmiter & Brinster, 1986). The ability to introduce and express cloned genes in transgenic systems allows investigation of the phenotypic effects of altered gene expression.

The phenotypic effects of altered gene expression by antisense RNA provide a unique opportunity to study the role of a specific gene function whose expression may be essential for normal development. However, to date, only one antisense mouse model has been reported (Katsuki et al, 1988). Antisense transgenic mice were produced with an antisense myelin basic protein (MBP) minigene construct. Several transgenic offspring of a founder transgenic mouse displayed the phenotype of the shiverer mutant mouse, which has an autosomal recessive mutation in the MBP gene (Roach et al, 1983; Molineaux et al, 1986). Further molecular analysis of the antisense shiverer phenotype showed the expression of antisense MBP mRNA and reduced amounts of endogenous MBP mRNA. The production of MBP and myelination in the central nervous system were also reduced.

In another study, Munir et al (1988) produced antisense transgenic mice with an antisense HPRT gene cloned to the mouse metallothionein promoter. Analysis of HPRT activity in the central nervous system of these transgenic mice, following zinc or cadmium induction, showed 50% reduction in animals heterogygous for the transgene.

The negation of gene expression by several different antisense HPRT constructs has been successfully demonstrated in early mouse embryos (table 4.6). Following the successful inhibition of gene expression by the inducible pMT-antiHPRT construct, antisense mice were produced with this sequence integrated into their genome in an attempt to create an inducible antisense system for study throughout development and in the adult mouse. The main purpose of creating an inducible antisense transgenic system was to study stage specific and tissue specific effects of antisense HPRT gene on endogenous HPRT gene expression. The preliminary results showed that 26% of live born individuals carried the antisense transgene (pMT-antiHPRT). The blood sample analysis of these transgenic mice showed no difference in overall HPRT activity compared with the normal non-transgenic mice (see table 4.8). However, from these preliminary results no final conclusion can be drawn. It is hoped that this project will be continued in the future.

## 4.4.9 Application of antisense technology

The potential uses of antisense inhibition could be many. The stably inherited expression of antisense RNA to inhibit the expression of a gene is a potentially useful tool for making a wide variety of mutant phenocopies. Antisense-induced phenocopies have distinct advantages over conventional mutants, because they may be created without altering the target gene. In addition, the conditional inhibition of target gene expression using inducible antisense genes provides a powerful means to elucidate the roles of specific gene

products in simple and complex biological systems and in precise developmental "windows".

Since the production of anti-message diminishes the expression of a variety of subsequently microinjected or endogenous genes, it may also be able to inhibit the activation of genes introduced by viral infection. In addition, this could facilitate the molecular analysis of viral gene activity and specific anti-messages may have therapeutic value in modulating the course of virus infection.

One of the many potential uses of antisense RNA may be extended to gene therapy. Further studies on potential applications of antisense RNA in both basic and applied science would throw light on gene regulation and its expression in development.

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