

Expression of injected HPRT minigene DNA in mouse embryos and its inhibition by antisense DNA

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Summary

We have used a highly sensitive biochemical microassay to monitor the expression of a cloned minigene for hypoxanthine phosphoribosyl transferase (HPRT, EC.2.4.2.8) in preimplantation mouse embryos. The mouse HPRT promoter and the mouse metallothionein promoter (MT-I) function equally well in embryos at the 2-cell stage whereas the viral SV40 promoter does not allow HPRT expression. Induced HPRT activity from the MT-I HPRT minigene con-

struct occurs in cleavage embryos cultured in the presence of cadmium. In contrast, negation of enzyme expression from the injected minigene DNA is mediated by simultaneous injection of HPRT antisense DNA.

Key words: preimplantation embryo, promoter function, HPRT minigene, antisense DNA, mouse embryo, cadmium, MT-I.

Introduction

The possibility of manipulating gene expression in early mammalian embryogenesis is important for the study of the genetic programme in development. The small amount of biological material of the mammalian egg and preimplantation embryo render molecular studies of the cleavage and blastocyst stages of development extremely difficult. Therefore, the study of specific promoter activity, promoter induction and antisense inhibition of gene activity during early development requires a sensitive assay to detect 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 (adenine phosphoribosyl transferase, EC.2.4.2.7) in a single egg, preimplantation embryo or even a single blastomere of the cleavage-stage embryo (Monk, 1987; Monk & Handyside, 1988; Monk *et al.* 1987).

The expression of a specific gene can be manipulated by using inducible promoters such as those directing the expression of mouse mammary tumor virus (MMTV) (Holt *et al.* 1986), interferon (Goodbourn *et al.* 1985), heat-shock protein (Wu, 1984; Topol *et al.* 1985) and metallothionein genes (Mayo

et al. 1982). Synthesis of mouse metallothionein-I (MT-I) mRNA is regulated by heavy metals and by glucocorticoid hormones (Mayo & Palmiter, 1981; Hager & Palmiter, 1981). When the MT-I promoter is linked to other DNA sequences, e.g. to the viral thymidine kinase gene, increased expression of these attached sequences is induced by the presence of cadmium (Mayo *et al.* 1982; Brinster *et al.* 1982).

The manipulation of gene expression using antisense RNA or DNA has been reported in various systems. Antisense RNA is able to form a duplex with its corresponding sense mRNA and thereby repress translation and expression of the specific gene product. Antisense blocking of gene expression has been shown by direct injection of antisense RNA into the cytoplasm of *Xenopus* oocytes (Harland & Weintraub, 1985; Melton, 1985; Wormington, 1986), *Drosophila* embryos (Rosenberg *et al.* 1985) and of mouse embryos (Bevilacqua *et al.* 1988). In other experiments, antisense RNAs generated *in vivo* from DNA vectors introduced into bacteria or eukaryotic cells, blocked the expression of a specific gene (Izant & Weintraub, 1984; Coleman *et al.* 1984; Petska *et al.* 1984; Kim & Wold, 1985; McGarry & Lindquist, 1986; Holt *et al.* 1986). For example, Izant & Weintraub (1984) showed a significant reduction in the expression of cloned TK gene injected into the nucleus

of TK⁻ mouse L cells by coinjecting a plasmid that directs the synthesis of antisense TK RNA.

The experiments reported in this paper involve the injection of HPRT minigene DNA into the male pronucleus of the fertilized mouse egg and assay of the resultant increase in HPRT activity over the basal level of endogenous HPRT activity in individual embryos. The simultaneous assay of endogenous APRT activity provides an internal standard which enables comparisons to be made between different embryos and different experimental procedures. In this system, induction of HPRT activity may be achieved by using an inducible promoter and repression of activity by simultaneous injection of antisense DNA. The HPRT and APRT double microassay serves as a reporter assay for the functioning of various promoters in the preimplantation embryo.

Materials and methods

Microinjection of embryos

Female F₁ (CBA × C57BL) mice were superovulated by intraperitoneal injection of 5 i.u. pregnant mare serum (Folligon) followed 45–48 h later by 5 i.u. human chorionic gonadotrophin (hCG, Chorulon, Intervet Lab. Ltd). They were then mated with F₁ males. Fertilized 1-cell eggs were collected from the oviducts 22–24 h post-hCG injection and the cumulus cells removed by treatment for 5 min with hyaluronidase (300 i.u. ml⁻¹). The eggs were washed and transferred to a glass chamber in M2 medium (Fulton & Whittingham, 1978). The paternal pronuclei of the eggs were injected with approximately 1500–2000 copies of supercoiled plasmid DNA. The injected eggs were transferred to droplets of M16 culture medium (Whittingham, 1971) under oil and cultured at 37°C in a 5% CO₂-in-air atmosphere.

HPRT and APRT microassay

Both injected and control uninjected embryos were harvested at the 2-cell, 5- to 8-cell, morula and blastocyst stages after 1, 2 and 3 days of culture, respectively. The embryos were washed in PB1.PVP (PB1 (Whittingham & Wales, 1969) with 4 mg ml⁻¹ polyvinylpyrrolidone (PVP) instead of albumin) and transferred in 5 µl of the 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 by Monk (1987).

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 (pDWM1Δ-638) was derived from the basic construct by deleting material between the *Bam*HI and *Bgl*II restriction sites (Fig. 1A). The expression vector, pMT142, was kindly provided by R. Palmiter. In this vector, the 0.77 kb *Eco*R-I/*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 (Fig. 1B). Plasmid pMT-HPRT (Fig. 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 pMT-antiHPRT (Fig. 1D) was constructed by deleting the 5' end of the HPRT minigene to -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. Plasmid pSV-HPRT (Fig. 1E) 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 Fig. 1C, thus bringing the HPRT minigene under the control of the SV40 promoter fragment.

Results

The entire HPRT gene is 33 kb 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 3 kb minigene functions efficiently in cultured mammalian cells (Melton *et al.* 1986). In the experiments reported below, we used a 5' deletional derivative of the basic minigene, pDWM1Δ-638, which 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 (Fig. 1). Further constructs were prepared (Fig. 1) in which the mouse HPRT promoter sequences from pDWM1Δ-638 were replaced by the promoter sequences of the mouse MT-I gene or of the simian virus, SV40. In addition, a construct was prepared in which the HPRT minigene was cloned in the antisense orientation with respect to the MT-I promoter.

To show transient expression of HPRT activity, male pronuclei of fertilized F₁ (CBA × C57BL) mouse eggs were injected with approximately 2 pl of 5 µg ml⁻¹ solution of supercoiled plasmids containing the HPRT minigene (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 standardized to the endogenous APRT activity in the embryos. One day after injection with the supercoiled HPRT minigene, pDWM1Δ-638, the 2-cell embryos showed an approximate twofold increase in HPRT activity and HPRT:APRT ratio compared with the control, uninjected, embryos (Table 1). The same twofold increase

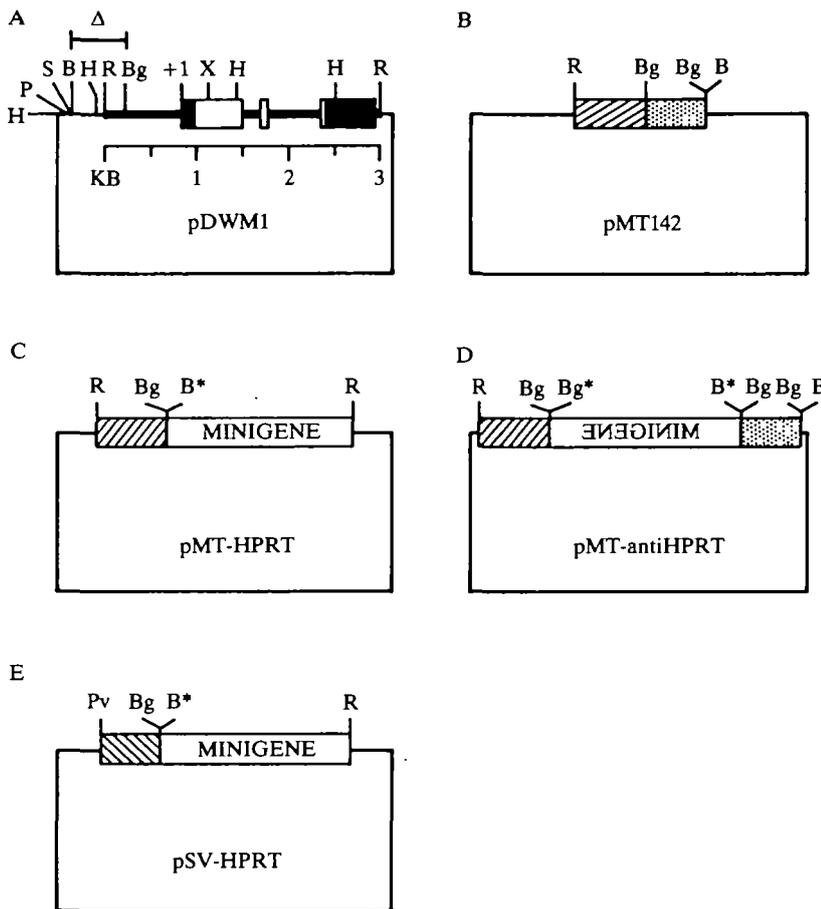


Fig. 1. Construction of plasmid vectors containing the HPRT minigene linked to different promoters. (A) pDWM1, the basic mouse HPRT minigene. Open boxes, HPRT coding sequence; closed boxes, untranslated regions; thick lines, flanking and intervening sequences; thin lines, vector (pUC8) sequence. The main transcriptional start site (+1) is indicated. (B) pMT-142, the human growth hormone gene driven by the metallothionein 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 promoter. (D) pMT-antiHPRT, the mouse HPRT minigene driven by the metallothionein promoter in the antisense orientation. (E) pSV-HPRT, the HPRT minigene cloned to the SV40 promoter. Restriction sites: B, *Bam*HI; 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).

in HPRT activity and HPRT:APRT ratio was observed when the HPRT minigene was transcribed from the mouse metallothionein-I promoter (pMT-HPRT). The increases in HPRT activity observed in the 2-cell embryos presumably result from transcription of the injected plasmids followed by translation into active enzyme protein. The endogenous APRT enzyme activities were similar in control and injected embryos.

When embryonic development was continued in culture beyond the 2-cell stage there was no further increase in HPRT activity and the injected embryos showed similar endogenous HPRT activities to control embryos at the 5- to 8-cell, morula and blastocyst

stages (Table 2). 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 three days of culture and, in these cases, the increase in HPRT activity was most marked (Table 2). No such increase in HPRT activity was observed in blocked 2-cell-stage control uninjected embryos during prolonged culture (data not shown).

The SV40 large T promoter has been linked to other gene sequences in a number of cases concerned with the study of gene expression in mammalian cells (Mulligan & Berg, 1980, 1981; Land *et al.* 1983), embryonic stem cells (Lovell-Badge *et al.* 1985) and

Table 1. Expression of HPRT activity at the 2-cell stage from injected HPRT minigene transcribed from the HPRT or MT-1 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 ± 9.6	0.69 ± 0.10

* Both HPRT and APRT enzymes are expressed in femtomoles h⁻¹ embryo⁻¹.

Table 2. Expression of HPRT activity from injected and endogenous HPRT genes during preimplantation development

Days in culture	Stage of development (no. embryos/sample)	HPRT*	
		Injected with pDWM1Δ-638 (no. samples)	Uninjected (no. samples)
1	2-cell (5)	103 ± 11 (3)	64 ± 4 (5)
2	5- to 8-cell (3)	368 ± 21 (3)	381 ± 23 (5)
3	morula (1)	930 ± 203 (4)	1150 ± 106 (7)
	blocked 1-cell (1)	7500 (1)	
	blocked 2-cell (1)	4420 (1)	

* HPRT enzyme activity is expressed in femtomoles h⁻¹ embryo⁻¹. (The average APRT activity is similar at all stages in injected and control embryos.)

production of transgenic mice (Adams *et al.* 1985). However, in our experiments, injection of two independent constructs of an HPRT minigene linked to the SV40 promoter (pSV-HPRT, Fig. 1) into mouse eggs failed to show increased HPRT activity in the embryos at the 2-cell stage (Table 1). Both pSV-HPRT constructs function in cultured cells (D.W.M., unpublished data). The failure of the pSV-HPRT constructs in 2-cell mouse embryos is in contrast to expression of *gpt* (guanosine phosphoribosyl transferase) from the SV40 promoter which was observed in 2-cell-stage embryos following pronuclear injection at the 1-cell stage (R.L.-B., unpublished data).

We investigated the induction of HPRT enzyme activity in preimplantation embryos after injection of the HPRT minigene linked to the mouse MT-I promoter into eggs and culture in the presence of cadmium. Preliminary experiments were carried out to determine the toxicity of varying concentrations of cadmium to morphological development of preimplantation embryos. Concentrations of cadmium above 10 μM were completely inhibitory to further development. Concentrations in the range of 3–10 μM allowed cleavage development to 2-cell stage and concentrations of 1 μM or less allowed normal development (data not shown). In previous work, Brinster

et al. (1982) reported induction of TK activity following injection of a TK gene fused with MT-I promoter into mouse eggs and culture for 22 h in 50 μM-cadmium. Since, in our hands, 50 μM-cadmium arrested development at the 1-cell stage, injected embryos were cultured either in medium without cadmium for 15 h followed by addition of 50 μM-cadmium to the resulting 2-cell embryos for a further 7 h, or continuously for 22 h in 1 μM-cadmium. The results for HPRT minigene expression are given in Table 3. The endogenous HPRT and APRT enzyme activities were not affected by the cadmium treatment alone. However, there was a marked and significant increase in HPRT activity in embryos injected with pMT-HPRT with cadmium treatment.

To investigate the effect of antisense HPRT DNA on enzyme activity expressed from exogenous HPRT minigene DNA, the HPRT minigene was injected together with a fivefold molar excess of antisense HPRT DNA. In control injected eggs, a fivefold molar excess of other DNA (pMT-TK, Brinster *et al.* 1982, or pMT-142, Fig. 1), in place of antisense DNA, was injected together with the HPRT minigene DNA. The resulting HPRT activity was again assayed at the 2-cell stage. In embryos injected with HPRT minigene, a fivefold excess of pMT-TK or pMT-142 DNA did not have any deleterious effect on cleavage to the 2-cell stage or on the increased expression of HPRT activity from the injected minigene. However, the presence of the antisense DNA reduced HPRT expression to the level found in uninjected controls (Table 4).

Discussion

The procedure of injection of HPRT minigene driven by different promoters 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 for promoter activity in early development and for induction as well as repression of gene activity in a single embryo.

Table 3. Induction of increased HPRT activity in embryos injected with pMT-HPRT by cadmium

Cadmium treatment μM	No. of samples (no. embryos/sample)	Injected			Uninjected control		
		HPRT*	APRT*	HPRT:APRT	HPRT*	APRT*	HPRT:APRT
50†	5 (2–3)	1144 ± 309	153 ± 24	7.29 ± 1.6	191 ± 13	152 ± 7	1.34 ± 0.11
1‡	4 (2–3)	1601 ± 654	119 ± 10	13.29 ± 6.0	197 ± 12	146 ± 9	1.38 ± 0.15
0	4 (1–3)	423 ± 124	138 ± 15	2.96 ± 0.69	188 ± 11	141 ± 9	1.34 ± 0.06

* Both HPRT and APRT enzymes are expressed in femtomoles h⁻¹ per 2-cell embryo.

† Cultured 15 h without cadmium followed by 7 h with cadmium.

‡ Cultured continuously for 22 h in cadmium.

Table 4. Inhibition of transient expression of HPRT activity from the injected HPRT minigene by simultaneous injection of antisense HPRT DNA

Expt number	Injected plasmid*	No. of embryos†	HPRT‡	APRT‡	HPRT:APRT
I	pMT-HPRT + pMT-TK	11	266 ± 55	110 ± 18	2.43 ± 0.23
	pMT-HPRT + pMT-anti-HPRT	8	133 ± 41	110 ± 28	1.29 ± 0.17
	Uninjected control	18	189 ± 14	163 ± 20	1.24 ± 0.14
II	pDWM1Δ-638	4	257 ± 59	215 ± 14	1.23 ± 0.20
	pDWM1Δ-638 + pMT-142	10	210 ± 48	175 ± 13	1.25 ± 0.39
	pDWM1Δ-638 + pMT-anti-HPRT	5	123 ± 14	187 ± 56	0.79 ± 0.15
	Uninjected control	18	101 ± 5	156 ± 12	0.67 ± 0.06
III	pDWM1Δ-638	10	133 ± 19	170 ± 11	0.83 ± 0.16
	pDWM1Δ-638 + pMT-142	4	162 ± 22	172 ± 7	0.95 ± 0.17
	pDWM1Δ-638 + pMT-anti-HPRT	4	56 ± 14	146 ± 30	0.41 ± 0.14
	Uninjected control	10	78 ± 8	136 ± 12	0.58 ± 0.09
IV	pDWM1Δ-638	10	186 ± 23	177 ± 2	1.06 ± 0.13
	pDWM1Δ-638 + pMT-142	8	163 ± 17	156 ± 21	1.07 ± 0.08
	pDWM1Δ-638 + pMT-anti-HPRT	13	105 ± 8	152 ± 14	0.71 ± 0.06
	Uninjected control	12	117 ± 5	165 ± 11	0.73 ± 0.06

* Fertilized eggs were injected with pMT-HPRT or pDWM1Δ-638 together with a fivefold excess of either pMT-anti-HPRT or, in controls, pMT-TK or pMT-142. (Plasmid pMT-TK was kindly provided by R. L. Brinster).

† Injected eggs were collected after 22 h and assayed singly.

‡ HPRT and APRT enzymes are expressed in femtomoles h⁻¹ embryo⁻¹.

The HPRT activity from the exogenously introduced gene is observed as an increase in activity over and above the endogenous HPRT activity levels. Endogenous HPRT and APRT activities are low in the egg and an increase is observed throughout preimplantation development. The increase in HPRT activity up until the 8-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). The significant increase in HPRT activity above the endogenous level following injection of the HPRT minigene is transient and is not observed beyond the 2-cell stage (Table 2). In rare cases, when the injected embryos remain blocked at the 1- or 2-cell stage, they show marked increase in HPRT activity (Table 2) even after 3 days of culture. The reason for this is not known. A similar large increase in *E. coli gpt* activity in embryos injected with cloned *gpt* sequences, and subsequently blocked in development, have been previously observed (R.L.-B., unpublished data).

Since the expression of the DNA injected into the fertilized egg may be observed only at the 2-cell stage (Table 2), all the manipulated embryos were subsequently monitored for gene activity at this stage. The efficacy of three different promoters were studied in 2-cell mouse embryos using the expression of HPRT as a reporter function. The mouse HPRT promoter and the uninduced mouse metallothionein-I promoter show an approximate twofold increase in HPRT activity and HPRT:APRT ratio indicating that both the promoters function equally well in the

early mouse embryo. In contrast, the viral SV40 promoter failed to function in mouse embryos at the 2-cell stage (Table 1), although these same constructs were expressed in cultured cells (D.W.M., unpublished data). It is possible that the specific sequences linked to the SV40 promoter actually affect the function of the promoter itself. In the experiments reported here, we do not know whether the failure of the SV40 promoter to give HPRT activity in embryos occurs at the level of transcription or translation.

In addition to heavy metal and glucocorticoid inducible 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 eggs and, therefore, the injected eggs were pulse treated with 50 μM-cadmium or cultured continuously in 1 μM-cadmium. Cadmium-treated embryos show a marked and significant increase in HPRT activities. The fact that treatment with 50 μM-cadmium at the 2-cell stage shows induction of HPRT activity in the injected embryos suggests that inducible HPRT minigene is still present at this time, at least 15 h after microinjection.

The use of antisense mRNA to block expression of specific gene products offers considerable potential as a means of investigating gene function *in vivo*. The results presented here (Table 4) suggest that a plasmid designed to produce antisense RNA may repress

the expression of exogenously introduced HPRT gene in mouse embryos. The fact that antisense HPRT DNA did not reduce the (basal) level of endogenous HPRT activity, which is attributable at this stage to maternally inherited enzyme and mRNA (Harper & Monk, 1983), suggests that the antisense DNA exerts its effect only within the nucleus. A series of recent studies in different systems have demonstrated that the expression of an antisense RNA complementary to a specific mRNA can effectively block the expression of specific genes (Coleman *et al.* 1983; Petska *et al.* 1984; Harland & Weintraub, 1985; Kim & Wold, 1985; Melton, 1985; Rosenberg *et al.* 1985; Holt *et al.* 1986; McGarry & Linqvist, 1986; Wormington, 1986; Bevilacqua *et al.* 1988). However, interference with gene expression in mammalian eggs by antisense DNA constructs has not previously been reported.

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 was 20- to 50-fold or greater (Wormington, 1986). When antisense DNA was used in transfection studies, as little as fivefold excess of antisense over sense lowered 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 our experiments, we used a fivefold excess of antisense DNA to sense DNA. Since we have shown the inducibility of pMT constructs, future experiments will test negation of gene activity by inducible HPRT antisense constructs used in reduced proportion to sense molecules injected into the embryos. These and future experiments will establish techniques for the manipulation (induction and repression) of expression of endogenous genes during preimplantation development of the mammalian embryo.

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(Accepted 1 August 1988)