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RNAs containing B2 repeated sequences are transcribed in the early stages of mouse embryogenesis

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An *in situ* hybridization technique was used to detect RNAs containing B2 sequences in the early mouse embryo. Accumulation of B2 sequences occurs early from the one cell stage. The level of B2 RNA decreases in the late two cell embryo, and then increases at the moment of second cleavage. In the blastocyst, inner cell mass cells contain more B2 transcripts than trophectoderm cells. In 7.5-day embryos the expression of B2 sequences is restricted to ectoderm and mesoderm. At all stages, transcription of the B2$^+$ strand is greater than B2$^-$ strand. We detected B2$^+$ RNAs in the nucleus and cytoplasm, whereas B2$^-$ RNAs were present only in the nucleus.

Key words: B2 family/embryogenesis/*in situ* hybridization/mouse/repetitive sequence

Introduction

The B2 sequences of the mouse are a family of repeated elements of 200 nucleotides, flanked with direct repeats on each side and present in 50 000–100 000 copies per genome (Kramerov *et al.*, 1979; Krayev *et al.*, 1982). The typical B2 consensus sequence contains promoter and terminator signals for RNA polymerase III and an (A+T)-rich region followed by an oligo(dA) stretch of 14–20 nucleotides (Krayev *et al.*, 1982). Variations among sequences in this family do not exceed 5–10% (Krayev *et al.*, 1982). B2 sequences have been detected outside single copy genes either at the 5′ (Kioussis *et al.*, 1981) or 3′ ends (Lalanne *et al.*, 1982) and inside introns (Barta *et al.*, 1981; Kioussis *et al.*, 1981; Steinmetz *et al.*, 1981; Vasseur *et al.*, 1985). Heavy nuclear RNA contains large amounts of B2 sequences, some of them found in double-stranded structures (Kramerov *et al.*, 1979). Cytoplasmic poly(A)$^+$ RNA contains transcripts in which the B2 sequences are either co-linear with various mRNA (Ryskov *et al.*, 1983), or are found as low mol. wt. heterogeneous (300–500 nucleotides) poly(A)$^+$ RNAs (Kramerov *et al.*, 1982). The latter class is believed to be transcribed by RNA polymerase III (Kramerov *et al.*, 1982). The transcription of cytoplasmic B2-containing RNAs seems to be tissue specific and associated with an embryonic or transformed phenotype: for instance, they are abundant in SV40-transformed fibroblast cells, and also in embryonal carcinoma (EC) cells but not in their differentiated derivatives (Bennett *et al.*, 1984; Murphy *et al.*, 1983; Rigby *et al.*, 1984). Since EC cells are known to be closely related to the multipotent cells of the early mouse embryo (Martin, 1980), we investigated the presence of B2 transcripts in early embryos, using *in situ* hybridization. The results presented here show that transcription of RNAs containing B2 sequences occurs very early and persists in pluripotential embryonic cells until day 7 of embryogenesis.

Results

We have performed *in situ* hybridizations on mouse embryos at various stages of development using B2 probes prepared from two independent B2 elements extracted either from the third intron of the *EndoA* gene (Vasseur *et al.*, 1985) or from the 3′ end of the *H2D* gene (Lalanne *et al.*, 1982). No differences related to the origin of the B2 probes were observed during these experiments. Since B2 sequences are highly repeated we checked that the hybridizations detected were strictly RNA dependent. Controls with RNase-treated preparations have always proved to be completely negative. Furthermore, the use of single-stranded DNA probes complementary to the (+) and (−) strands of the B2 sequence gave very different patterns of hybridization (see below). This also allowed us to characterize the polarity of the transcripts. Below we refer to the poly(A)-containing strand as the B2$^+$ RNA and to the complementary strand as the B2$^-$. Table I qualitatively summarizes the amounts of B2$^+$ and B2$^-$ RNA sequences present in the cytoplasm and the nuclei of embryonic cells from the one-cell to the late primitive streak stages.

B2 transcripts in one-cell embryos

RNA containing B2 sequences was detected in unfertilized mouse eggs. At this step, the labeling of the oocyte was low, but uniform all over the cytoplasm and the nucleus. Only B2$^+$ RNAs were present (Figure 1). After fertilization, but before fusion of the pronuclei, the intensity of hybridization increased (Figure 1). Although B2$^+$ RNAs were detected in both nucleus and cytoplasm, the labeling in the nuclei was greater than in the cytoplasms (Figure 2; Table I). Moreover, we found that one of the pronuclei was always more labeled than the other, indicating a difference in their content of B2$^+$ transcripts, but it was not possible to distinguish the maternal or paternal origin of the pronuclei (Figure 2). B2$^-$ RNAs were present only at background level (Figure 1, Table I). The polar body contained some B2$^+$ transcripts with a labeling identical to that of the cytoplasm.

Two-cell embryos

At the two-cell stage, between 24 and 40 h post-fertilization, several types of labeling were observed (Figure 2). Some embryos were heavily labeled in the nucleus and cytoplasm of both cells while others exhibited only a low diffuse cytoplasmic reaction. Both strands of the B2 sequence were present in the cellular RNA at this stage, but B2$^-$ RNAs were confined to the nuclei and in much lower amount (10–100 times) than B2$^+$ RNAs (Figure 1, Table I). Three-cell stage embryos containing one 1/2 blastomere and a 2/4 couplet (formed from the early division of the other 1/2 cell) were occasionally encountered. The nuclei of the 2/4 couplet were consistently labeled, in contrast to the nucleus of the larger 1/2 blastomere which remained negative (Figure 2). Most of the polar bodies observed contained B2$^+$ RNAs at a level identical to the signal detected in the cell's nuclei.

Morulae and blastocysts

At the four-cell stage, the four blastomeres were heavily labeled,

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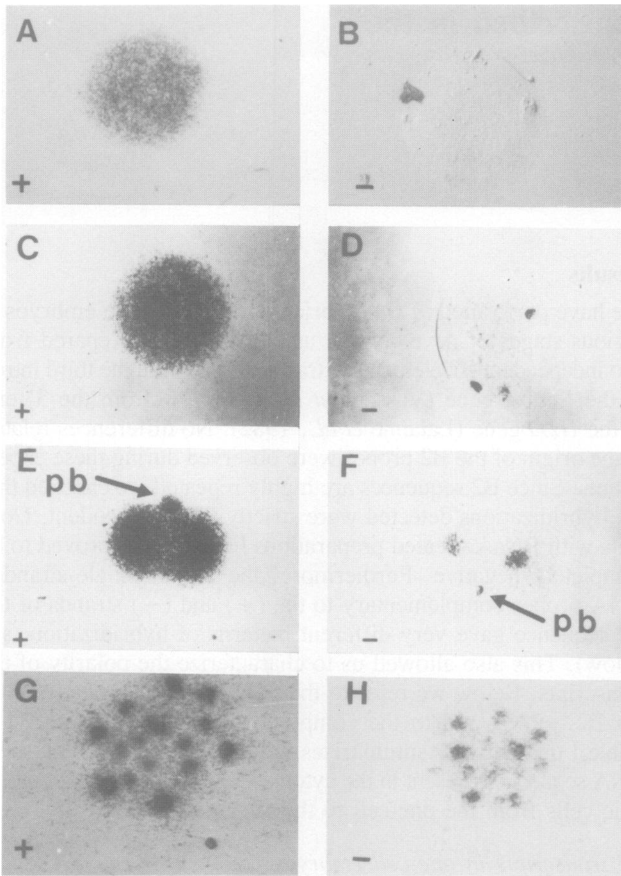

Fig. 1. Comparative expression of B2$^+$ and B2$^-$ transcripts from the one-cell to the morula stage. Hybridization of single-stranded probes in non-fertilized **(A,B)**, fertilized **(C,D)**, two-cell embryos **(E,F)** and morula **(G,H)**. B2$^+$ RNAs are revealed in **A, C, E, G** and B2$^-$ RNAs in **B, D, F, H** (5 x 10^5 c.p.m./slide for the (+) strand; 3 x 10^5 c.p.m./slide for the (−) strand; exposure time was 8 days for **A, C, E, G** and 2 weeks for **B, D, F, H**; pictures were taken in bright field illumination). (x150). pb: polar body.

mostly in the nuclei (Figure 2). Some embryos (< 10%) of this stage, in which only one or two blastomeres were labeled, were also observed. The hybridization patterns of the eight-cell embryos were more homogeneous. All the cells were labeled with the same intensity, and always more in the nucleus than in the cytoplasm (Figure 2, Table I). The RNAs in these stages were mainly B2$^+$ RNAs (Figure 1, Table I). An increase in B2$^-$ RNA as compared with early cleavage stages was noticeable from this stage on.

In the blastocyst, hybridization of the B2 transcripts presented three characteristic features: (i) both nuclei and cytoplasm were labeled; (ii) the amount of B2$^-$ RNA was markedly increased; (iii) hybridization to B2$^+$ and B2$^-$ probes tended to decrease in the trophectoderm cells of expanding blastocysts while the inner cell mass cells remained heavily labeled (Figure 3A, Table I).

Post-implantation embryos

In 7.5-day old embryos (late primitive streak stage) the B2 probes hybridized in a tissue-specific manner. The ectoderm and mesoderm cells were labeled (in their nuclei and cytoplasm), while the extra-embryonic and embryonic endoderm were negative (Figure 3, B and C). Some of the decidual cells were also labeled, mostly in their nuclei and these positive cells were frequently arranged in couplets. In the decidua, as well as in the embryonic ectoderm and mesoderm, B2$^+$ RNAs were more abundant than B2$^-$ RNA.

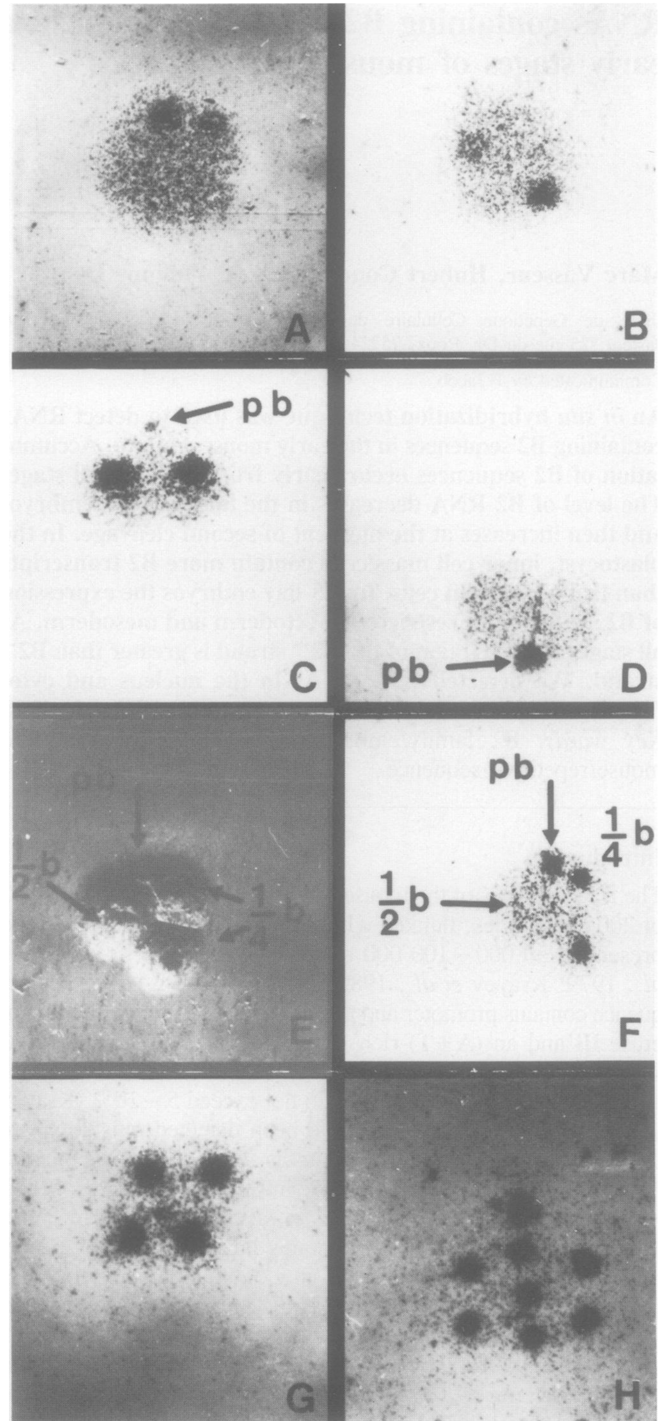

Fig. 2. Expression of B2 transcripts from the fertilized egg to the eight-cell stage. Hybridizations were performed with nick-translated probes (8 x 10^5 c.p.m./slide; 10 days of exposure) (x200). **(A,B)** Two examples of labeling of one-cell stage embryos. **(C,D)** Two examples of the labeling observed in two-cell stage embryos. **(E,F)** Three-cell stage embryo shown in phase contrast **(E)** and bright field illumination **(F)**. pb: polar body; 1/2 b: two-cell stage blastomere; 1/4 b: four-cell stage blastomere. **(G)** Four-cell stage embryo. **(H)** Eight-cell stage embryo.

Discussion

We have observed that RNAs containing B2 sequences are present in low levels in unfertilized mouse eggs, and that their levels significantly increase during early embryogenesis. Such an accumulation of RNA containing interspersed repetitive sequence has already been reported to occur in the oocyte of other organ-

Table I. Qualitative recapitulation of the level of hybridization to $B2^+$ and $B2^-$ RNA sequences during early mouse embryogenesis

Embryonic stages	$B2^+$ RNA		$B2^-$ RNA	
	Nucleus	Cytoplasm	Nucleus	Cytoplasm
Unfertilized oocyte	+	+	−	−
One-cell	++	+	(±)	−
Early two-cell	+++	++	+	−
Late two-cell	+	+	−	−
Four-cell	+++	+	−	−
Morula	+++	++	+	−
Blastocyst:				
Trophectoderm:				
early	+++	++	+	−
late	−	+	−	−
ICM:				
early	+++	++	++	−
late	+++	++	++	−
7.5-day:				
visceral endoderm (embryonic and extra embryonic)	−	−	−	−
mesoderm	+++	++	+	−
ectoderm	+++	++	+	−

We evaluated the amount of labeling in the cytoplasm and in the nucleus simply by visual inspection. Comparisons were made between hybridizations performed in the same conditions (same probe, same radioactive input, same exposure time). Evaluations of the intensities of labeling are reported in this table using a scale of (−) to (+++). (−): no hybridization; (+) to (+++): low to maximum signals.

isms (Anderson *et al.*, 1982), but the role of such transcripts remains unknown. The presence of an increased level of RNA containing B2 sequences in the pronuclei of the fertilized oocyte is consistent with a *de novo* transcription at this stage (Clegg and Piko, 1982). In the polar body expelled after fertilization, a level of hybridization, roughly identical to the signal detected in the egg cytoplasm, is observed. At the two-cell stage, the polar body is heavily labeled with the $B2^+$ but not with the $B2^-$ probe. Thus, it is possible that fertilization has activated transcription of RNAs containing B2 sequences in both the embryonic nuclei and polar bodies. This is the first observation of a transcriptional activity in a polar body.

Some of the two-cell embryos are intensely labeled in both cells while others are not. In the three-cell stage, the late 1/2 blastomere is negative while the two other 1/4 blastomeres are labeled. This suggests that the transcription of B2 RNAs is turned off in late 1/2 blastomeres to be resumed at the time of the second cleavage. Thus, there may be two waves of B2 transcription, coinciding with the first and second cleavages. Labeled and unlabeled two-cell embryos would then correspond to early and late two-cell stages. Such an interpretation implies that B2 RNAs synthesized around the time of the first cleavage are significantly degraded during the 15 h of the two-cell stage. The observation of specific transcriptional events temporally related to cell division has already been reported for cells cultured *in vitro* (Linzer and Nathans, 1983). The difference in the B2 contents observed between trophectoderm and ICM cells could be related to the different commitments of these two tissues. This interpretation is supported by the observation of 7.5-day embryos in which B2 sequences are expressed in ectoderm and mesoderm, but not in endoderm cells. This suggests that regulation of B2 transcription is not only related to cell division, but could be associated also with particular programs of differentiation. *In vitro*, while

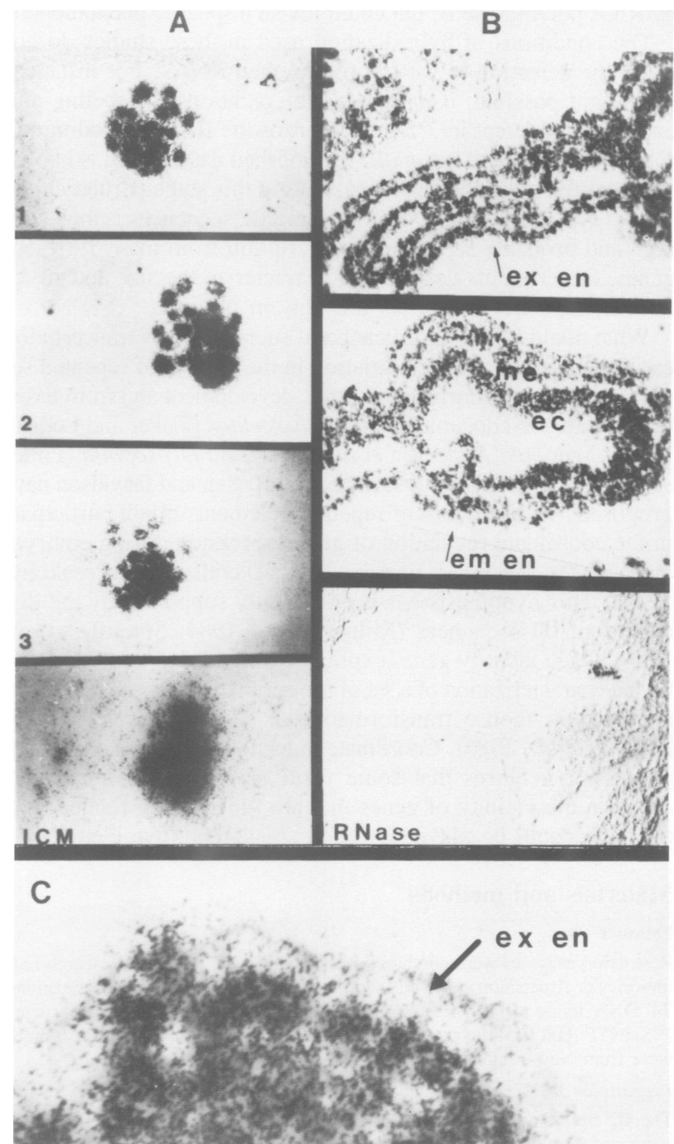

Fig. 3. **(A)** 3.5−4 days blastocysts hybridized to nick-translated B2 probe as in Figure . Three different types of pattern of expanding blastocysts are presented **(1, 2, 3)** (x150). ICM: hybridization of an isolated inner cell mass from 3.5-day blastocyst (x225). **(B)** Transcription of B2 sequences in a 7.5-day mouse embryo. Slightly oblique section through a 7.5-day embryo hybridized to a single-stranded B2 + probe (6 x 10^5 c.p.m./slide; 6 days of exposure) (x150). ec: ectoderm; me: mesoderm; ex en: extra embryonic endoderm; em en: embryonic endoderm. RNase: RNase pre-treated hybridization. **(C)** Magnification of the extra embryonic endoderm of a 7.5-day embryo hybridized as in **B**. (x500).

cytoplasmic B2 RNAs are heavily transcribed in EC cells, their amount is significantly reduced after differentiation. The F9 cell line, in particular, contains a high level of B2 small transcripts which become almost undetectable after differentiation into endoderm (Murphy *et al.*, 1983). This result parallels our observations in the embryo.

The differences in the hybridization pattern observed between the (+) and (−) strands show that the $B2^+$ strand is the most abundant at all stages studied, and possibly the only one to be present in the cytoplasm. The quantitative difference between transcription of the two strands suggests that this expression is not the result of a random transcription of B2 elements dispersed around or inside single copy genes and co-transcribed occasionally

by RNA polymerase II, but could reveal a specific phenomenon.

The conditions of hybridization used in these studies do not allow the detection of weakly represented RNAs. For instance, it was not possible, using the same conditions of labeling and exposure, to detect in 7.5-day embryos the RNAs encoding the *EndoA* protein (Vasseur *et al.*, unpublished data) which is known to be expressed in the endoderm cells at this stage (Brûlet *et al.*, 1980). The amount of RNA containing B2 sequences is thus very high and probably represents transcription from many different genes. Experiments designed to characterize the size and structure of these B2 transcripts are now in progress.

What could be the significance of such a type of transcription early in embryogenesis? Variation in the pattern of repeated sequence expression during embryonic development and from tissue to tissue are well documented in *Dictyostelium* (Zuker and Lodish, 1981), *Xenopus* (Anderson *et al.*, 1982) and *Drosophila* (Potter *et al.*, 1979). Considering such results, Britten and Davidson have proposed that a network of repetitive elements might participate in the coordinate regulation of gene expression during embryogenesis (Davidson and Britten, 1979; Davidson and Posakony, 1982). This hypothesis has been recently supported by the discovery of ID sequences (Milner *et al.*, 1984; Sutcliffe *et al.*, 1984) which identify genes expressed only in rat brain cells, and by the characterization of a set of transcripts sharing a B2 element, activated in mouse transformed cells (Brickell *et al.*, 1983; Murphy *et al.*, 1983). Coordinate induction or repression of gene expression requires that some form of homologous sequence exists in the vicinity of genes that are functionally related, and this role could be played by such small repetitive elements.

Materials and methods

Enzymes

Restriction enzymes were purchased from Boehringer, Bethesda Research Laboratories or Amersham and used according to the manufacturers' specifications. T4 DNA ligase, S1 nuclease and DNA polymerase were from Boehringer. [^{35}S]dNTP (600 Ci/mmol) were from Amersham and [^{35}S]dNTP (1200 Ci/mmol) were from New England Nuclear.

Preparation of ^{35}S-labeled probes

The B2 probes were prepared either from a 160 bp long *Pst*I-*Pst*I fragment recovered from the pH2D3 plasmid containing H2D cDNA (a gift of J.L.Lalanne, Lalanne *et al.*, 1982) or from a 310 bp long *Eco*RI-*Bam*HI fragment excised from the third intron of the *EndoA* gene (Vasseur *et al.*, 1984) and subcloned into pEMBL 9 (Dente *et al.*, 1983). Radioactive nick-translated probes were obtained by labeling the purified insert with [^{35}S]dATP and dCTP at a specific activity of 600 Ci/mmol (Rigby *et al.*, 1977). In order to prepare single-stranded probes, the fragment prepared from the *EndoA* gene was cloned into M13 mp8 and mp9 vectors (Messing and Vieira, 1982). 500 ng of a recombinant M13 was annealed to 5 ng of 17-nucleotide sequencing primer (Amersham) for 2 h at 56°C in 10 μl of a buffer containing 10 mM Tris pH 7.4, 10 mM $MgCl_2$ and 500 mM NaCl. The Klenow DNA polymerase (0.5 U), 0.5 μM dGTP and dTTP, and up to 100 μCi of [^{35}S]dCTP and dATP (1200 Ci/mmol) were added. The mixture was incubated at 25°C for 30 min followed by a 15 min chase after addition of 0.5 μM dATP and dCTP. The synthesized DNA was then restricted by *Eco*RI (for mp8 recombinant) or *Bam*HI (for mp9 recombinant) which cut once outside the inserted B2 sequence. After denaturing the DNA, the single-stranded digested fragment was fractionated on a 5% polyacrylamide sequencing gel (Sanger *et al.*, 1978) and recovered by elution with 0.5 M NH_4OAc, 10 mM $MgCl_2$, 1 mM EDTA, 0.1% SDS at 37°C (Nicolas and Berg, 1983). After addition of single-stranded carrier salmon DNA (300 μg/ ml), the probe was ethanol precipitated and resuspended in the hybridization mixture at the desired concentration.

Preparation of embryos and in situ hybridization

Embryos were obtained from crosses of ♀ F1 C57BL/6-BALB/c or F1 C57BL/6-DBA/2 x ♂ F1 C57BL/6-DBA/2. Pure mouse strains came from inbred stocks kept at the Institut Pasteur. Preparation of pre-implantation embryos was performed as described (Brûlet *et al.*, 1985) and deposited on glass slides treated according to Haase *et al.* (1984). Hybridization was performed according to Hafen *et al.* (1983) except that the concentration of single-stranded salmon sperm DNA was 300 μg/ml, oligo(dT)$_{15}$ was added at a concentration of 50 μg/ml, and that the hybridization solution contained 2.5 x Denhardt's mixture. Hybridization was carried out for 24 h at 37°C and washes were performed at 37°C. Control preparations were digested with DNase-free RNase at a concentration of 50 μg/ml for 2 h at 37°C. Kodak NTB-2 emulsion was used for autoradiography which was performed at −20°C for 4 days to 2 weeks.

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