Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 2054–2058, April 1985
Developmental Biology

Spatial distribution of transcripts of the long repeated ETn sequence during early mouse embryogenesis

(endogenous retrovirus/RNA·DNA *in situ* hybridization/long terminal repeat/early transposon)

PHILIPPE BRÛLET, HUBERT CONDAMINE, AND FRANÇOIS JACOB

Unité de Génétique Cellulaire du Collège de France et de l'Institut Pasteur, 25, rue du Dr. Roux, 75724 Paris Cedex 15, France

Contributed by François Jacob, November 19, 1984

ABSTRACT **RNA·DNA *in situ* hybridization revealed a high level of ETn ("early transposon") transcripts in the pluripotent cell lineage of the 3.5- to 7.5-day mouse embryo. Some extra-embryonic ectoderm derivatives also show a high level of ETn transcripts at these stages. Older embryos (8.5 days and later) have a uniform low level of ETn transcripts.**

In the course of studies on molecular aspects of mouse blastocyst formation, we have identified a new family of moderately repeated, dispersed sequences, which were designated ETn, for "early transposon" (1).

By differential screening of a cDNA library constructed from embryonal carcinoma (EC) cell mRNA, an ETn cDNA clone was first isolated on the criterion that it contains a sequence specifically transcribed in EC cell lines. Using the cDNA sequence pMS3 as a probe, a genomic DNA recombinant was randomly selected and a 6-kilobase DNA fragment that spans the entire ETn sequence was subcloned (pMAC-2 plasmid) and used to further study the ETn family. This sequence is bordered by direct repeats several hundred base pairs (bp) long. ETn RNA was shown by R-loop analysis to be colinear with the randomly selected genomic fragment in pMAC-2 (1). A distinctive feature of the ETn family is that its sequences are transcribed at a high level in undifferentiated EC cell lines but not in any of a variety of differentiated cell lines tested (1).

In another paper, we shall report a detailed analysis of the two direct long terminal repeats (LTRs). Our results show that the 5′ and 3′ ends of the RNA are located within the LTRs. The LTR structure is essentially identical to that of retroviral LTRs, but their sequence seems to differ from any LTR sequence known so far.

We have investigated the presence of ETn transcripts in the early mouse embryo by *in situ* RNA·DNA hybridization. Our results show that the pattern of expression previously detected *in vitro* in cell lines parallels the situation that prevails in normal embryogenesis: the ETn sequences are transcribed at a high rate in early pluripotent cell lineages, from blastocyst inner cell mass to embryonic ectoderm of the late primitive-streak stage (between 3.5 and 7.5 days of embryonic development), and also in extra-embryonic ectoderm layers. In contrast, a low level of transcription occurs in most other committed tissues both before and after gastrulation.

MATERIALS AND METHODS

Cells. The cell lines used in this study were described previously and were cultured under standard conditions (1).

Enzymes. Restriction enzymes were purchased from Boehringer Mannheim, Bethesda Research Laboratories, or New England Biolabs and used according to the manufacturers' specifications. T4 DNA ligase, nuclease S1, and DNA polymerase were from Boehringer Mannheim. [α-^{32}P]-dNTPs (800 Ci/mmol; 1 Ci = 37 GBq) were from Amersham. ^{35}S-labeled dNTPs (1200 Ci/mmol) were from New England Nuclear.

Single-Stranded Radioactive DNA Probes. One microgram of a recombinant molecule in M13, containing the 5′ or 3′ LTR, was hybridized to 4 ng of 15-nucleotide sequencing primer (New England Biolabs) in 10 μl of 7 mM $MgCl_2$/7 mM Tris Cl, pH 7.5/0.5 M NaCl. The Klenow fragment of DNA polymerase I (0.5 unit) and 10 μCi of [α-^{32}P]dATP were used to synthesize the complementary strand at 30°C for 30 min, followed by a 10-min "chase" with unlabeled dATP. After ethanol precipitation, the DNA was cut with a restriction enzyme outside the inserted LTR DNA, for nuclease S1 mapping. After the digested DNA was denatured at 95°C for 3 min in 50% dimethyl sulfoxide, the fragments were fractionated in a 1.2% agarose gel (Bethesda Research Laboratories Minigel). The radioactive single-stranded fragments were located by autoradiography and electrophoretically recovered. After one phenol and one chloroform extraction, the fragments were recovered by ethanol precipitation.

Nuclease S1 Mapping. RNA and poly(A)$^+$ RNA were prepared from EC cells as previously reported (2). The labeled single-stranded DNA fragment (5×10^4 to 5×10^5 cpm) was mixed with the RNA (2 μg of poly(A)$^+$ RNA or up to 100 μg of unfractionated RNA) and ethanol-precipitated. The nucleic acids were resuspended in 18.4 μl of 40 mM Pipes, pH 6.4/1 mM EDTA/80% formamide and heated for 5 min at 75°C. Hybridization with the DNA was performed by incubating the sample at 37°C for 5–15 hr in the same buffer containing 0.4 M NaCl. The reaction was stopped by adding 300 μl of cold S1 buffer (0.3 M sodium acetate, pH 4.8/0.2 M NaCl/4.5 mM $ZnSO_4$) containing 3000 units of nuclease S1; the samples then were incubated 2–10 hr at 20°C, 37°C, 50°C, or 62°C. After ethanol precipitation, the pellets were dissolved in 25 μl of 0.3 M NaOH and heated 30 min at 70°C, and the solution was neutralized with 25 μl of 0.3 M HCl and 0.5 M Tris Cl, pH 8.3. Then 5 μg of tRNA was added, and the solution was extracted once with phenol, and once with chloroform and then ethanol-precipitated. Finally, the pellets were suspended in 25 μl of 95% (vol/vol) formamide/dye mixture and heated for 3 min at 95°C; 2.5-μl samples were electrophoresed in 8% acrylamide sequencing gels. Autoradiography was usually for 2 hr. Size markers were provided by the four sequencing-reaction "ladders" of various clones.

***In Situ* Hybridization.** *Preparation of embryos and embryo sections.* Embryos used were from the following crosses: ♀ C57BL/6-BALB/c × ♂ BALB/c or ♂ 129/So; ♀ C57BL/6.CBA × ♂ C57BL/6.DBA/2; ♀ C57BL/6.BALB/c or C3H/He.129/So × ♂ C57BL/6.DBA/2. No systematic differences related to the genotype of the embryos were ob-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: LTR, long terminal repeat; EC, embryonal carcinoma.

served in the results of the experiments. Pure-line mice came from inbred stocks kept at the Institut Pasteur. All hybrids were obtained by crossing individuals from these stocks.

Preimplantation embryos, from prepuberal females in which superovulation had been induced by standard techniques, were flushed at appropriate times into PB1 medium (3) at room temperature, deprived of their zona pellucida by brief exposure to Tyrode's solution (pH 2.5) (4), fixed in paraformaldehyde [3% (wt/vol) in phosphate-buffered saline (P_i/NaCl), 20 min at room temperature], rinsed twice in P_i/NaCl and placed successively in cold 30% and 60% ethanol. Microdrops of 60% ethanol containing one or a few embryos were deposited on glass slides treated according to Haase *et al.* (5) and air-dried, which ensured spreading and attachment of the embryos. These were post-fixed in paraformaldehyde as above, rinsed in P_i/NaCl, and dehydrated in a graded series of ethanol (2 min each in 30%, 60%, 80%, 94%, and 100% ethanol). After air-drying, the slides were stored at −20°C for up to 2 weeks.

Blastocyst inner cell masses, obtained by immunosurgery (6) with an anti-EC cell line rabbit antiserum (a gift of M. H. Buc, Institut Pasteur) as a source of anti-mouse antibodies and rabbit complement (a gift of M. Pla, Institut National de la Santé et de la Recherche Médicale U 93, Hôpital Saint-Louis, Paris), were treated like whole preimplantation embryos.

Postimplantation embryos came from spontaneous matings; 6.5–9.5 day embryos were fixed inside their decidua in paraformaldehyde (4% in P_i/NaCl) at 4°C for about 20 hr, rinsed in P_i/NaCl, and then incubated in 0.5 M sucrose/P_i/NaCl for 24 hr at 4°C. Older embryos were dissected free from their extra-embryonic membranes and treated in the same way. The fixed, sucrose-treated preparations then were blotted dry on filter paper, quick-frozen in liquid nitrogen, and mounted in Tissue-Tek II (Miles). Sections (8 μm) were made in a cryostat at −22°C, collected on glass slides prepared as above, placed for 1 min on a hot plate (50°C), and air-dried at room temperature for about 1 hr. The sec-

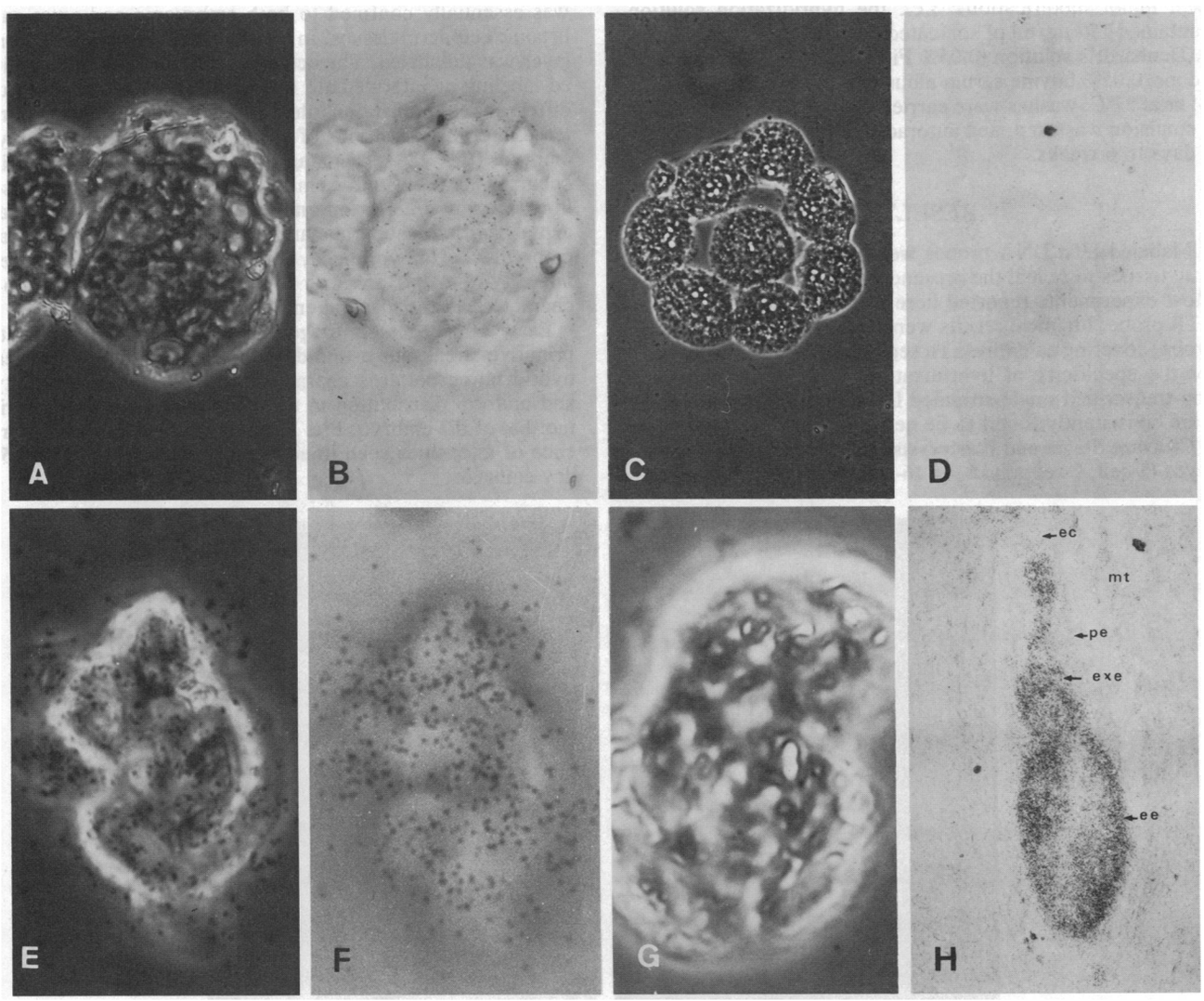

FIG. 1. Transcription of ETn LTR in preimplantation embryos (*A–G*) and 6.5-day egg cylinder (*H*). (*A* and *B*) Blastocysts (3.5 days) hybridized to an ^{35}S-labeled 5′ LTR single-stranded probe (2×10^5 cpm per slide; 6 weeks of autoradiographic exposure) corresponding to the transcribed LTR strand. (×228.) (*C* and *D*) Eight-cell 2.5-day morula, same hybridization as in *A* and *B*. (×137.) (*E* and *F*) An inner cell mass isolated from a 3.5-day blastocyst, same hybridization as in *A* and *B*. (×520.) (*G*) An inner cell mass from 3.5-day blastocyst, hybridized to an ^{35}S-labeled 5′ LTR single-stranded probe corresponding to the untranscribed LTR strand (2×10^5 cpm per slide; 6 weeks of exposure). (×715.) (*H*) Parasagittal section through a 6.5-day embryo hybridized to single-stranded 5′ LTR probe (2×10^6 cpm per slide; 2 weeks of exposure). ec, Ectoplacental cone; pe, proximal endoderm; exe, extra-embryonic ectoderm; ee, embryonic ectoderm; mt, maternal tissue. (×87.) (*A–G*) Embryos were from F_1 C57BL/6 × BALB/c females mated to F_1 C57BL/6 × DBA/2 males. (*H*) Embryo is from an F_1 C57BL/6 × CBA female mated to a male of the same genotype. *A*, *C*, *E*, and *G* show phase contrast. *B*, *D*, *F*, and *H* show bright-field illumination.

tions then were post-fixed, dehydrated, and kept at −20°C as above.

Preparation of tissues for hybridization. The permeabilization and Pronase treatment described by Hafen *et al.* (7) was used without any change. After Pronase digestion, embryos and embryo sections were again fixed and dehydrated as described above.

DNA probes used for hybridization. Double-stranded DNA (1 μg) was nick-translated in the presence of 100 μCi of 5′-[α-[^{35}S]thio]dGTP and 5′-[α-[^{35}S]thio]dATP (1200 Ci/mmol) at 14°C for 90 min. After addition of 150 μg of sonicated salmon sperm DNA, the probes were purified successively by centrifugation through a G-50 medium Sephadex column, phenol/chloroform extraction, and ethanol precipitation. The specific activity of the purified probes usually ranged from 10^8 to 2×10^8 cpm per μg.

Single-stranded DNA was synthesized as explained above from an M13 recombinant by using 10 μCi of 5′-[α-[^{35}S]-thio]dGTP and was purified by agarose gel electrophoresis.

Hybridization. The protocol of Hafen *et al.* (7) was used with minor modifications: i.e., the hybridization solution contained 150 μg/ml of sonicated salmon sperm DNA in 2.5 × Denhardt's solution (0.05% Ficoll/0.05% polyvinylpyrrolidone/0.05% bovine serum albumin); hybridization was for 24 hr at 37°C; washes were carried out at 37°C; Kodak NTB-3 emulsion was used; and autoradiography was at −20°C for 4 days to 6 weeks.

RESULTS

^{35}S-labeled ETn DNA probes were applied to early embryonic tissues to reveal the presence of complementary RNA. Most experiments reported here were done with a 5′ or 3′ LTR probe. Identical results were obtained when a pMAC-2 probe, covering an entire ETn sequence, was used. Controls for the specificity of hybridization were carried out with non-transcribed single-stranded DNA from LTR probes and were consistently found to be negative.

Cleavage Stages and Blastocysts. When early-cleavage embryos (1-cell, 2-cell, and 8- to 16-cell stages) were examined for their ability to hybridize with ETn LTR DNA, very little hybridization was found to occur (Fig. 1 *C* and *D*). In contrast, a plasmid carrying an actin cDNA sequence (a gift from M. Buckingham, Institut Pasteur) hybridized heavily with 8–16 cell morulae, indicating that hybridizable RNA, when present, is indeed accessible to the DNA of the probe under the conditions used (data not shown). Whole-blastocyst spreads showed a somewhat denser concentration of hybridization grains (Fig. 1 *A* and *B*). However, when blastocyst inner cell masses were dissected immunosurgically and processed in the same way as whole blastocysts, they appeared to hybridize with a much greater intensity (Fig. 1 *E* and *F*). Control hybridizations with untranscribed single-stranded LTR DNA showed that inner cell masses remain negative in this case (Fig. 1*G*), as do morulae and whole blastocytes (not shown).

Egg-Cylinder and Primitive-Streak Stages. An intense labeling was seen in cryostat sections of late egg-cylinder (6.5-day) embryos (Fig. 1*H*), comparable to the labeling exhibited by F9 EC cells grown in aggregates (not shown). The label was essentially confined to both embryonic and extra-embryonic ectoderm tissues. In contrast, the visceral endoderm layer was unlabeled. The ectoplacental cone region exhibited labeling of intermediate intensity. The clearest cases of differential hybridization intensities, however, were encountered in cryostat sections of primitive-streak stage (7.5-day) embryos (Fig. 2). At that stage, an intense hybridization still occurred in the definitive embryonic ectoderm layer and also in extra-embryonic ectoderm derivatives lining the ectoplacental cavity. Mesodermal areas (both embryonic and extra-embryonic) also hybridized, although with a distinctly lower intensity. Finally, an even lower degree of hybridization characterized the visceral endoderm layer.

Early Organogenesis Stages. Tissue sections from post-primitive-streak stage (8.5-day and later) embryos yielded hybridization patterns characterized by both low intensity and uniform distribution in the various organ anlagen (primordia) of the embryo. Fig. 3 exemplifies the typical difference of intensities seen in ectoderm layers of 7.5- and 8.5-day embryos.

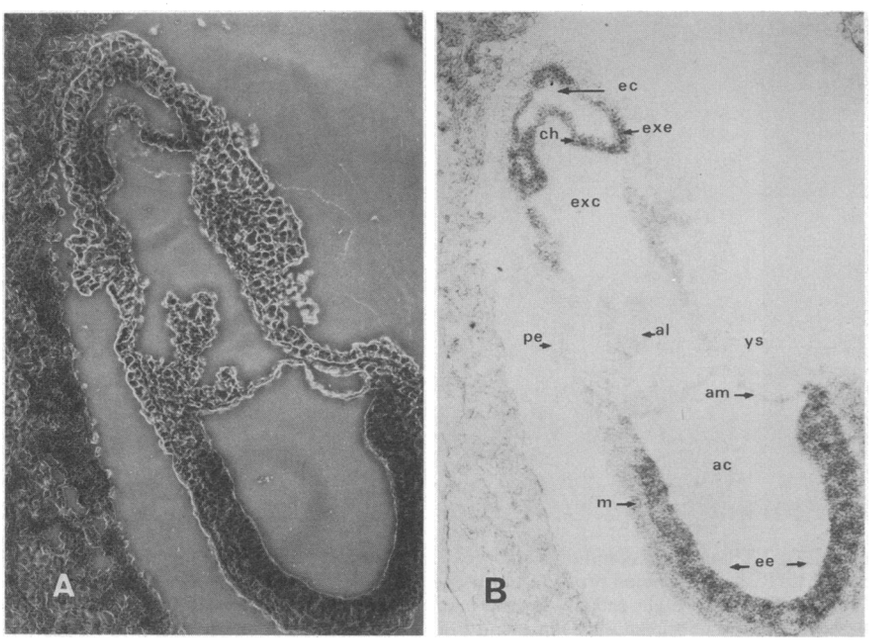

FIG. 2. Transcription of ETn LTR in a 7.5-day mouse embryo. Slightly oblique section through an embryo (from F_1 C57BL/6 × CBA female mated to F_1 C57BL/6 × DBA/2 male) hybridized to a single-stranded (transcribed strand) ^{35}S-labeled 5′ LTR probe (2×10^6 cpm per slide; 2 weeks of exposure). (*A*) Phase contrast. (*B*) Bright field. ec, Ectoplacental cavity; exe, extra-embryonic ectoderm; ch, chorion; exc, exocoelomic cavity; pe, proximal endoderm; al, allantois; ys, yolk sac; am, amnion; ac, amniotic cavity; m, mesoderm; ee, embryonic ectoderm. (×101.)

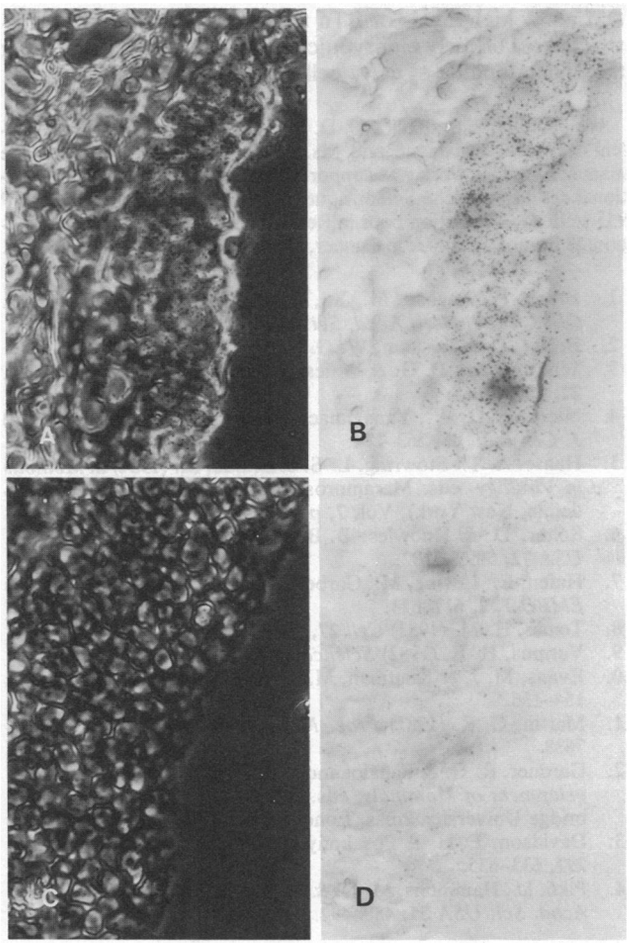

FIG. 3. Transcription of ETn LTR in the ectoderm of 7.5-day (*A* and *B*) and 8.5-day embryos (*C* and *D*). The embryos were from F_1 C57BL/6 × BALB/c females mated to F_1 C57BL/6 × DBA/2 males. (*A* and *C*) Phase contrast. (*B* and *D*) Bright field. Hybridization was to a single-stranded (transcribed strand) ^{35}S-labeled 5′ LTR probe (2×10^5 cpm per slide; 4.5 weeks of exposure). Note the low hybridization level in mesoderm below the ectoderm of 7.5-day embryos (*A* and *B*). (×180.)

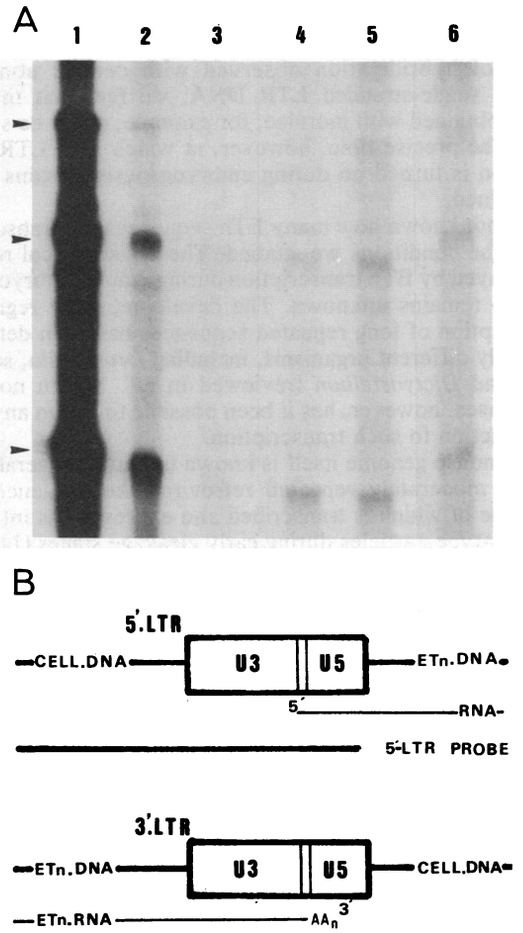

FIG. 4. Nuclease S1 analysis of embryonic ETn RNA. (*A*) The single-stranded 5′ LTR probe was hybridized to unfractionated cellular RNA for 14 hr at 37°C and then digested with nuclease S1 for 5 hr at 37°C. The products were analyzed in 8% polyacrylamide gels. Autoradiography was for 14 hr at −20°C. Arrows indicate three DNA fragments protected by ETn RNA. Their sizes correspond to the sizes of the LTR, U3, and R.U5 domains of the probe. Lanes 1, 2, and 3: 100 μg, 10 μg, and 1 μg of PCC4 RNA, respectively. Lanes 4 and 5: 100 μg of 9.5-day and 10.5-day embryo RNA. Lane 6: 100 μg of TDM-1 trophoblastoma RNA. (*B*) Diagram of ETn transcription unit. U3, U5, and R (between U3 and U5) are regions of the LTR mentioned in the text. The single-stranded 5′ LTR probe obtained from an M13mp8 recombinant molecule (see *Materials and Methods*) comprised 670 bases upstream of the LTR and the entire LTR itself except for the last 12 bases of its 3′ end. AA_n, poly(A) tail.

To estimate the ETn RNA that might persist during these stages, RNA was extracted from whole 9.5- to 13.5-day embryos and analyzed by nuclease S1 mapping (see *Materials and Methods*). The amounts of specific RNA thus detected were compared to those present in undifferentiated EC cells (Fig. 4). The relative amount of ETn RNA present in total RNA from 9.5- to 13.5-day embryos could thus be estimated to be <5% of that present in the RNA from the PCC4 EC cell line. As a comparison, the same S1 mapping was performed with RNA extracted from TDM-1, a trophoblastoma cell line. The amount of specific RNA detected was roughly equivalent to that obtained from 9.5-day embryos (Fig. 4).

DISCUSSION

A structural analysis to be presented elsewhere has established that the two LTRs bordering the long moderately repeated ETn sequences are identical to retroviral LTRs. In particular, ETn sequences are transcribed in EC cells into an RNA that exhibits the same pattern of transcript ends among U3, R, and U5 domains (8, 9). The results of RNA·DNA *in situ* hybridization experiments presented here indicate that ETn transcription also occurs in normal embryonic cells.

The pluripotent cell lineage, which is segregated in the blastocyst inner cell mass and persists up to the formation of the primitive streak, clearly appears to be the compartment with the most intense ETn transcription. The abundance of ETn RNA initially observed in EC cells (1) thus corresponds to a feature of normal embryogenesis, since EC cells are equivalent to close descendants of inner-cell-mass cells (10, 11). However, ETn RNA also appears to be abundant in extra-embryonic ectoderm tissues, indicating that ETn transcription does not characterize any one cell lineage in the early mouse embryo, since embryonic and extra-embryonic ectoderms are believed to derive from two distinctly committed lineages in the blastocyst (12).

The low levels of *in situ* hybridization in post-primitive-streak embryos could be correlated with low amounts of correctly initiated ETn RNA present in tissues from 9.5- to 13.5-day embryos. The general picture that emerges from both *in situ* hybridization and nuclease S1 mapping experiments is that, after day 8, ETn transcription levels decrease in all tissues of the embryo to <5% of the level in undifferentiated

EC cells. Such a correlation is still lacking for preimplantation embryos. Nevertheless, in view of the low background levels of hybridization observed with control non-transcribed single-stranded LTR DNA, we feel that the few grains obtained with morulae, for example, might be significant. The precise time, however, at which ETn LTR transcription is turned on during embryogenesis remains to be determined.

It is not known how many ETn sequences are transcribed under the conditions we studied. The physiological role, if any, played by ETn transcription during mouse embryogenesis also remains unknown. The developmentally regulated transcription of long repeated sequences has been detected in widely different organisms, including *Drosophila*, sea urchin, and *Dictyostelium* (reviewed in ref. 13). In none of these cases, however, has it been possible to assign any precise function to such transcription.

The mouse genome itself is known to harbor several families of moderately repeated retrovirus-like sequences, at least one of which is transcribed and expressed as intracisternal A-type particles during early cleavage stages (14). No significant homology was found between ETn LTR and LTR sequences from retroviruses or from other retroviral-like sequences in the mouse genome (1). It is, however, clear that knowledge of the complete nucleotide sequence of the ETn internal region will be necessary to exclude homologies between the various families.

There remains a possibility that the ETn transcription detected in our experiments is but the first step of a purely "selfish" process of retroviral-particle production taking place mainly in the pluripotent lineage of the early embryo. This might provide an explanation for the amplification of that sequence in the germ line, since germ cells would not yet have been segregated when this hypothetical retroviral cycle would occur. However, elucidation of the mechanisms that allow high levels of ETn transcription to occur in a restricted set of early embryonic lineages might yield clues for the understanding of early cell commitment processes.

We should like to thank Ms. D. Boullier and M. Maury for excellent technical assistance and Ms. M. Compain for preparing the manuscript. This work was supported by grants from the Centre National de la Recherche Scientifique (LA 269, ATP 955 153, and ATP 955 189), the Fondation pour la Recherche Médicale, the Ligue Nationale Française contre le Cancer, and the Fondation André Meyer.

1. Brûlet, P., Kaghad, M., Xu, Y.-S., Croissant, O. & Jacob, F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5641–5645.
2. Brûlet, P. & Roskam, W. G. (1983) *Gene* **22**, 59–68.
3. Whittingham, D. G. & Wales, R. G. (1969) *Aust. J. Biol. Sci.* **22**, 1065–1068.
4. Nicolson, G. W., Yanagimachi, R. & Yanagimachi, H. (1975) *J. Cell Biol.* **66**, 263–274.
5. Haase, A. T., Stowring, L. S. & Brahic, M. (1984) in *Methods in Virology*, eds. Maramorosch, K. & Koprowski, H. (Academic, New York), Vol. 7, pp. 189–226.
6. Solter, D. & Knowles, B. B. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 5099–5102.
7. Hafen, E., Levine, M., Garber, R. L. & Gehring, W. J. (1983) *EMBO J.* **2**, 617–623.
8. Temin, H. M. (1981) *Cell* **27**, 1–3.
9. Varmus, H. E. (1982) *Science* **216**, 812–820.
10. Evans, M. J. & Kaufman, M. H. (1981) *Nature (London)* **292**, 154–156.
11. Martin, G. R. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638.
12. Gardner, R. G. & Papaioannou, V. E. (1975) in *The Early Development of Mammals*, eds. Balls, M. & Wild, A. E. (Cambridge University Press, London), pp. 107–133.
13. Davidson, E. H. & Posakony, J. W. (1982) *Nature (London)* **297**, 633–635.
14. Pikó, L., Hammons, M. D. & Taylor, K. D. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 488–492.