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Expression of the cytokeratin endo A gene during early mouse embryogenesis

(nuclease S1 mapping/*in situ* hybridization)

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ABSTRACT **Expression of cytokeratin endo A has been analyzed during mouse blastocyst formation and embryonal carcinoma cell differentiation. To study the regulation of endo A expression, nuclease S1 mapping experiments have been performed on RNA extracted from two-cell to 7.5-day embryos. Low levels of endo A mRNA begin to be detectable in eight-cell embryos. The amount of this mRNA increases at the blastocyst stage, suggesting that endo A expression is regulated at the mRNA level during blastocyst formation. At this stage, *in situ* hybridization studies show that endo A mRNA is present in the trophectoderm but not in the inner cell mass. In 7.5-day embryos, endo A mRNAs are also detectable in the endoderm layer and in the amnion.**

During mouse embryogenesis, the transition from the eight-cell morula to the blastocyst stage is characterized by the appearance of two different cell types, the inner cell mass and the trophectoderm (1, 2). The inner cell mass contains totipotent cells, whereas the trophectoderm is composed of differentiated epithelial cells (1, 3). Later, a second epithelial cell type appears on the surface of the inner cell mass, forming the primitive endoderm (4). These two epithelia express cytokeratin intermediate filaments (5–8). One of the major components of these intermediate filaments is the protein named cytokeratin endo A, also referred to as cytokeratin A (5, 7, 9, 10). Endo A can be first detected in the eight-cell morula; it increases significantly during blastocyst formation (10). At this stage, it is found in the trophectoderm but not in the inner cell mass (5). Later in development, endo A is expressed in endoderm cells but neither in pluripotent embryonic ectoderm nor in mesoderm (11, 12). Endo A provides therefore a valuable marker for the study of the molecular events involved in early epithelial differentiation. *In vitro*, endo A is found in trophoblastoma and endodermal cell lines (5, 11, 13) but not in embryonal carcinoma (EC) cells. Retinoic acid, which triggers F9 EC cells to differentiate into endoderm-like cells (14), induces the synthesis of endo A (13).

To study the regulation of endo A expression during early steps of embryogenesis, we have recently isolated genomic clones encoding this protein (15–17). We have analyzed the amount of endo A mRNA synthesized during *in vitro* EC cell differentiation and during early embryogenesis using an ultrasensitive nuclease S1 mapping procedure. The transcripts were localized by *in situ* hybridization.

MATERIALS AND METHODS

Mice. The inbred congeneic strains BALB/c, DBA/2, and C57BL/6 maintained at the Institut Pasteur were used. For embryo analysis, matings between (*i*) female F_1(C57BL/6 × CBA/J) and male F_1(C57BL/6 × CBA/J) or (*ii*) female BALB/c and male DBA/2 were used.

Embryos. Embryos (two-cell-stage, eight-cell-stage, or blastocysts) were recovered from virgin superovulated 3- to 5-week-old female mice that had been mated 3 or 4 days before. Embryos were washed extensively in a phosphate buffer medium and kept at −70°C before RNA extraction.

Cells. F9 and PCC3 are EC cell lines (18, 19) that were cultured in an undifferentiated state under standard conditions (20). F9 cells were induced to differentiate into endoderm-like cells by using retinoic acid at 0.2 μM as described by Strickland and Mahdavi (14). TDM1 is a trophoblastoma cell line (19). SVT2 is a transformed BALB/c mouse fibroblastic cell line (21).

RNA Extraction. RNA was extracted from adult organs and cell lines as described by Auffray and Rougeon (22). RNA extraction from batches of embryos was performed as described by Clegg and Piko (23).

Nuclease S1 Mapping and RNA Transfer Blots. The *Sma* I–*Sma* I fragment, which contains the cap site of endo A mRNA, has been described (17). It was used to prepare single-stranded probes that were uniformly labeled to a high specific activity by using a modification of the procedure described by Battey *et al.* (24). Hybridizations, nuclease S1 digestions, and polyacrylamide gel analysis were performed as described (17). RNA transfer blots were prepared according to Derman *et al.* (25) and were hybridized with 10^6 cpm/ml of a single-stranded probe labeled to a specific activity of 2×10^9 cpm/μg. To quantitate the relative amount of mRNA, the bands on the autoradiograms were scanned with an integrator/photometer Vermon and the surfaces under the peaks were measured. The densitometric values for the different bands were calculated by comparing bands of the same autoradiogram.

***In Situ* Hybridizations.** *In situ* hybridization studies were performed as described in Brûlet *et al.* (26). The 5′ single-stranded probe used in these experiments has been described above; 5×10^5 to 10^6 cpm were placed on each slide.

RESULTS

Analysis of Endo A mRNA in EC Cells and in Their Differentiated Derivatives. To study endo A mRNA by using the nuclease S1 mapping procedure, a 295-base-pair-long *Sma* I–*Sma* I fragment (Fig. 1) that encompasses the 5′ end of the mRNA (16) was used as a probe. This fragment does not hybridize to any of the other members of the cytokeratin multigene family, thus providing a specific probe for endo A (16). This fragment was subcloned into M13 mp8 to prepare single-stranded probes that were hybridized to total or

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Abbreviation: EC, embryonal carcinoma.

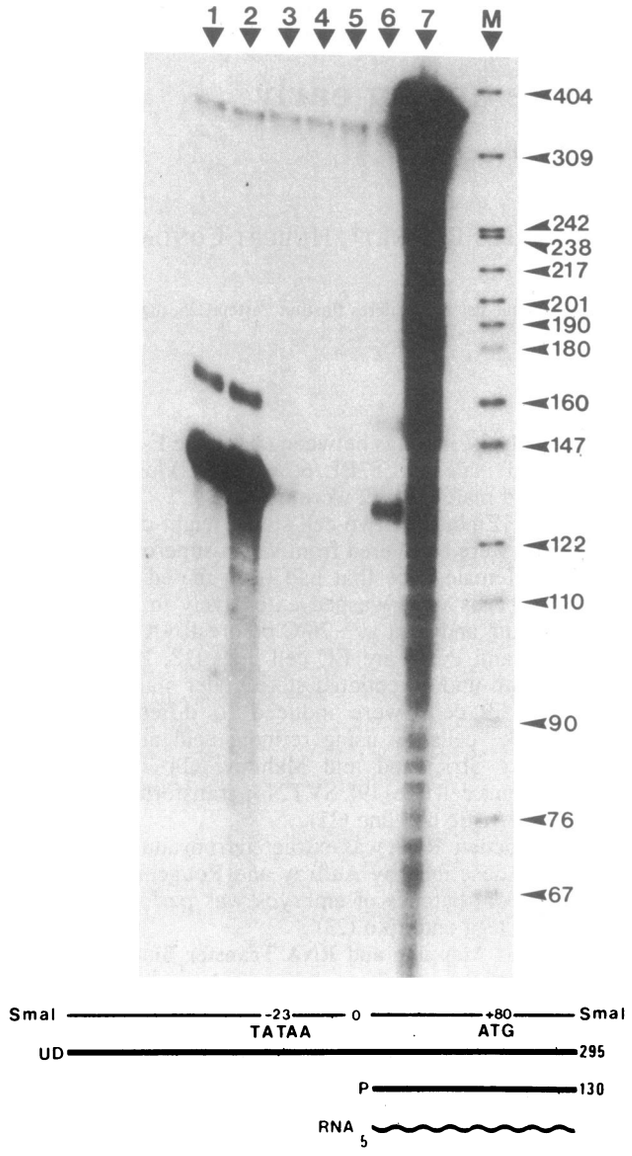

FIG. 1. Nuclease S1 mapping of endo A mRNA in cultured cells. Structures of the undigested probe (UD) and of the fragment protected from nuclease S1 digestion by the mRNA are schematized below the autoradiogram. Illustrated is the hybridization of 10 μg of total RNA extracted from TDM1 cells (lane 1), F9 cells treated with retinoic acid for 96 hr (lane 2), F9 cells (lane 3), 3T3 cells (lane 4), SVT2 cells (lane 5), and PCC3 cells (lane 6). Lane 7, nonhybridized and undigested probe. Lane 8, size markers (shown in bases). The 50-base-pair difference between the theoretical length of the undigested probe and the size observed on the gel is due to the M13 mp8 polylinker and primer sequences that were removed during nuclease S1 digestion. The autoradiogram was exposed for 19 hr at −80°C.

poly(A)-RNA extracted either from TDM1, F9, or retinoic acid-treated F9 cells. Hybridization with RNA from TDM1 trophoblastoma cells yielded a 130-base-long fragment, which corresponds to the major (90%) cap site of the mRNA, and a minor 153-base band (Fig. 1), which indicates an initiation (10%) in the TATAA box (17). Although the endo A protein is not expressed in EC cells, very low levels of endo A mRNA were detected in undifferentiated PCC3 and F9 cells (Fig. 1). A 96-hr retinoic acid treatment of F9 cells caused a 50-fold increase in the amount of endo A mRNA. The 5′ ends of the transcripts in PCC3 and F9 cells were the same as in TDM1 cells. The presence of endo A mRNA was also investigated in fibroblast cell lines, such as 3T3 and SVT2, in which the cytokeratins are not expressed. No endo A mRNA was detected in these cells even after prolonged exposure. Thus, the block of endo A gene expression appears to be complete in fibroblasts, whereas low levels of endo A mRNA can be detected in populations of EC cells that potentially can differentiate into endoderm cells. This low level is likely to be due to the presence of a few differentiated derivatives in the population of EC cells.

Analysis of Endo A mRNA in Early Preimplantation Embryos. Total RNAs extracted from 2-cell embryos, 8-cell embryos, and preimplantation blastocysts of 60 cells were subjected to nuclease S1 mapping with the probe described above labeled to very high specific activity. No transcripts were detected in RNA extracted from 1200 2-cell embryos (Fig. 2*A*). Endo A mRNA was detected in 1000 8-cell embryos and its level increased significantly at the blastocyst stage (only 250 embryos were needed for each hybridization). Because of the very low amount of material available, the concentration of RNA used in these experiments was not measured. Piko and Clegg (27) have found that an 8-cell embryo contains 0.70 ng of total RNA and a 32-cell blastocyst contains 1.50 ng. According to these data, and assuming that the total amount of RNA in a blastocyst is proportional to the number of cells, the increase in the amount of endo A mRNA from the 8-cell to the 64-cell embryo was estimated to be 10-fold.

Some high molecular weight bands can be observed in these nuclease S1 mapping experiments. However, the size and distribution of such bands varied from one experiment to another, while the 130-base fragment corresponding to the major cap site of endo A mRNA was constant. It is likely that these bands are nuclease S1 digestion artefacts that become apparent during the overexposure of the gel necessary to detect low levels of transcripts in 8-cell morulae.

Analysis of Endo A mRNA in Postimplantation Embryos. Endo A mRNA was studied in 7.5-day embryos freed of trophoblast and ectoplacental cone. The amount of endo A mRNA in one 7.5-day embryo (which contains ≈1 μg of total RNA) was lower by a factor of 3 than that found in 250 blastocysts (which contain 700 ng of total RNA) (Fig. 2*B*). This relative decrease can be explained since the signal observed is likely to originate mainly from the endoderm layer, which expresses endo A (11). Experiments using *in situ* hybridization showed that endo A mRNA was present in this layer and also in the amnion (see below).

In the course of these experiments, RNA extracted from adult epithelia (liver or kidney) was used as positive controls. The amount of endo A mRNA in these tissues is lower than that in the trophoblastoma cell line TDM1 (lower by factors of 10 and 15 in liver and kidney, respectively) (Fig. 2 *B* and *C*). To evaluate endo A mRNA content of endoderm cells, total RNA was extracted from yolk sac (which is composed of visceral and parietal endoderm and of extraembryonic mesoderm) dissected from 10-day embryos and was found to contain a high level of endo A mRNA, equivalent to the amount found in TDM1 cells (Fig. 2*D*).

Size of the Endo A mRNA in Cultured Cells and in Early Embryos. An 18S mRNA encoding endo A has been described in TDM1 cells (15). To determine whether RNAs extracted from blastocysts, 7.5-day embryos, and EC cells had the same size, RNA transfer blots were prepared and hybridized with the single-stranded *Sma* I–*Sma*·I probe tagged with ^{32}P-labeled dNTPs to a specific activity of 2×10^9 cpm/μg. After a 3-week exposure of the autoradiograms, an 18S RNA was detected in blastocysts and in 7.5-day embryos (Fig. 3). An RNA of the same size was also observed in PCC3 cells, in F9 cells treated with retinoic acid, in TDM1 cells, and in adult tissues. In the case of F9 cells, poly(A)$^+$ RNA had to be used to detect a signal. By using RNA transfer analysis, the relative amounts of endo A RNA found in these cells was

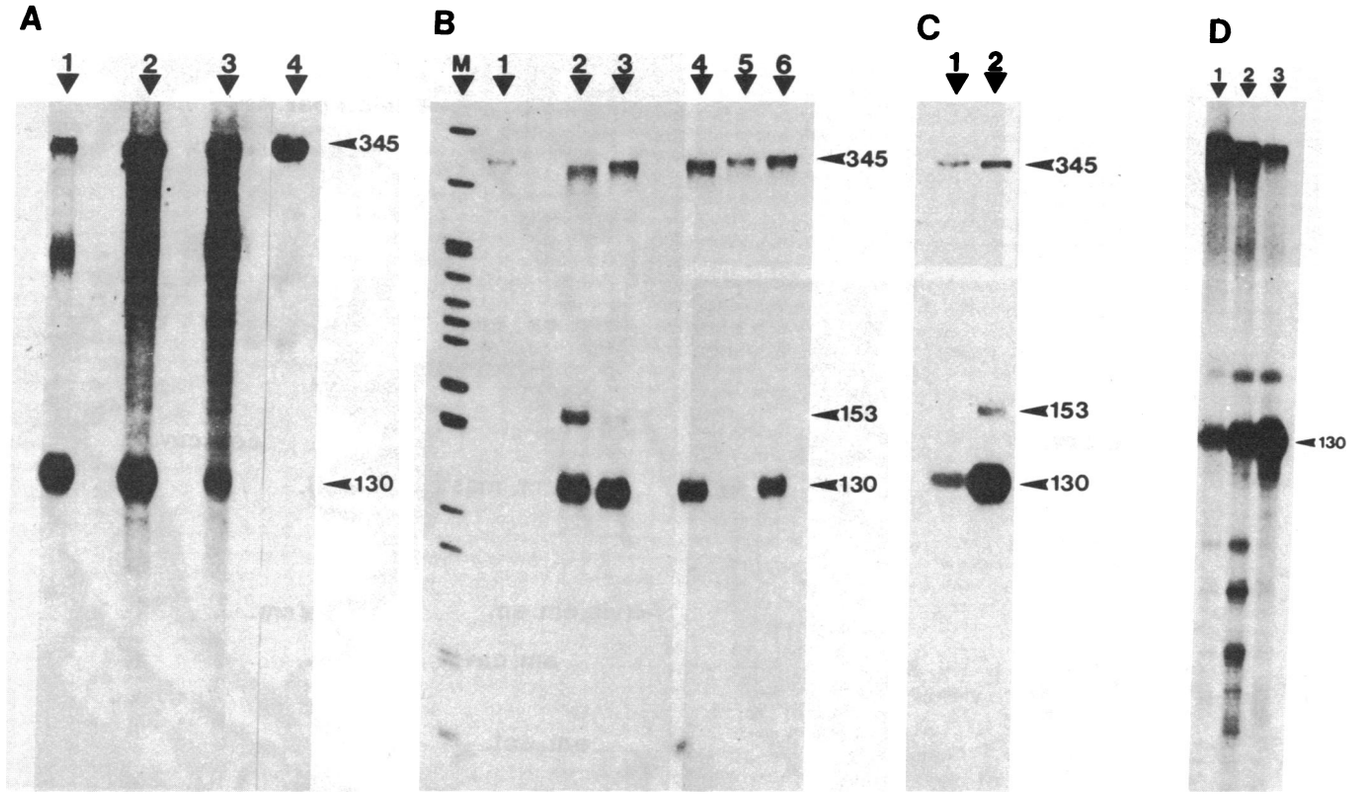

FIG. 2. Nuclease S1 mapping of endo A mRNA in mouse early embryo and in adult tissues. The probe used was the same as that used in Fig. 1. (*A*) Hybridizations with total RNA extracted from 250 blastocysts (lane 1), 500 blastocysts (lane 2), 1000 8-cell embryos (lane 3), and 1200 2-cell embryos (lane 4). Exposure was 120 hr at −80°C. (*B*) Hybridization with total RNA. Lane 1, control, 10 μg of *Escherichia coli* tRNA; lane 2, kidney, 10 μg; lane 3, liver, 10 μg; lane 4, RNA from three 7.5-day embryos; lane 5, RNA from one 7.5-day embryo; lane 6, RNA from 250 blastocysts. Exposure was 19 hr at −80°C. Size markers (shown in bases) are the same as in Fig. 1. (*C*) Hybridization with 10 μg of total RNA extracted from liver (lane 1) and TDM1 (lane 2). Exposure was 10 hr at −80°C. (*D*) Hybridization with 5 μg of total RNA extracted from a 10-day embryo freed of extraembryonic membranes (lane 1), yolk sac dissected from a 10-day embryo (lane 2), TDM1 cells (lane 3). Exposure was 20 hr at −80°C.

the same as that found with the nuclease S1 procedure. Therefore, the endo A transcripts detected by nuclease S1 mapping probably correspond to full-length endo A mRNA and not to abortive transcripts. In the case of eight-cell morulae, the scarcity of material available did not allow size analysis by the RNA transfer blotting technique.

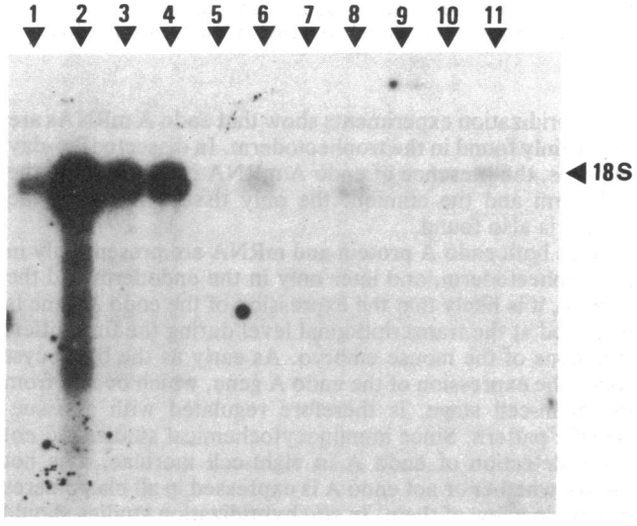

FIG. 3. RNA transfer blot analysis of RNA extracted from 5 μg of poly(A)$^+$ RNA from F9 cells (lane 1) and 25 μg of total RNA from TDM1 cells (lane 2). Lanes 3–9, 5 μg of RNA was used, extracted from TDM1 (lane 3), F9 cells treated with retinoic acid for 96 hr (lane 4), F9 cells (lane 5), PCC3 (lane 6), 3T3 (lane 7), liver (lane 8), and kidney (lane 9). Lane 10, total RNA extracted from one 7.5-day embryo; lane 11, total RNA extracted from 250 preimplantation blastocysts. Exposure was 21 days at −80°C.

Localization of Endo A Transcripts in Preimplantation Blastocyst and in 7.5-Day Embryo. The results presented above show that the amount of endo A mRNA increases during blastocyst formation. However, the cellular localization of such transcripts remains unknown, although immunocytochemical studies have demonstrated that the protein is present mostly in the trophectoderm cells (5, 10). To localize endo A transcripts, *in situ* hybridization experiments were performed on preimplantation embryos by using single-stranded probes derived from the *Sma* I–*Sma* I fragment tagged with ^{35}S-labeled dATP and dCTP by primer extension. No significant labeling was found in eight-cell embryos (data not shown) with this procedure, suggesting that this method is not sensitive enough to reveal the low level of transcripts found by nuclease S1 mapping. In preimplantation blastocysts, we observed a diffuse labeling (more intense, for some of them, in the periphery of the embryo), suggesting that the transcripts might be confined mostly to the trophectoderm (Fig. 4*A*). This hypothesis was confirmed by *in situ* hybridizations performed directly on immunosurgically isolated inner cell masses that were not significantly labeled under the same experimental conditions (Fig. 4*B*). Thus, the endo A transcripts detected in blastocysts are likely to be produced only in the trophectoderm. The same *in situ* hybridization experiments were performed on 7.5-day embryos. A very specific labeling pattern was observed. The embryonic and extraembryonic visceral endoderms were

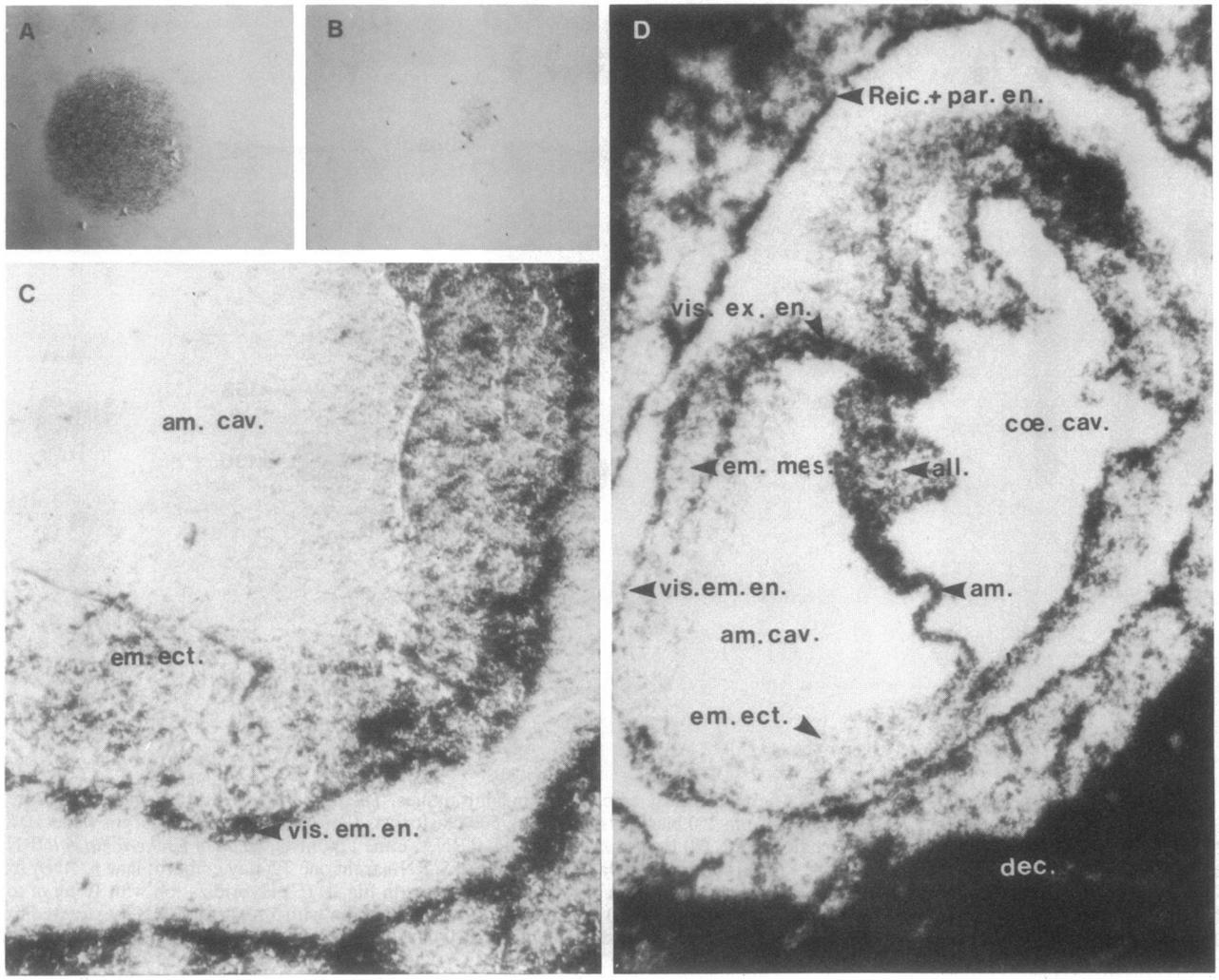

FIG. 4. *In situ* hybridization of preimplantation blastocyst (*A*), isolated inner cell mass (*B*), and 7.5-day embryo (*C* and *D*) (5×10^5 cpm per slide). The probe used for these experiments is described in the legend to Fig. 1 and was labeled to high specific activity by using the four ^{35}S-labeled dNTPs (New England Nuclear). Exposure was 60 days (*A* and *B*) or 110 days (*C* and *D*). (*C*) A magnification of the embryonic part of the embryo. vis. em. en., Visceral embryonic endoderm; em. ect., embryonic ectoderm; dec., decidua; am. cav., amniotic cavity; am., amnion; em. mes., embryonic mesoderm; all., allantois; vis. ex. en., visceral extraembryonic endoderm; coe. cav., coelomic cavity; Reic. + par. en., Reichert's membrane and parietal endoderm.

labeled but not the ectoderm or the mesoderm (Fig. 4*C*). Moreover, the amnion was also found to be labeled (Fig. 4*D*) and immunofluorescence experiments have confirmed the presence of the endo A protein in this layer (data not shown). The decidua, which is composed of epithelial cells, was heavily labeled (Fig. 4*D*).

DISCUSSION

We have shown that the amount of endo A mRNA, initially low in multipotential cells, increased significantly during F9 cell differentiation and blastocyst formation. In the two-cell mouse embryo, no endo A transcripts were detected by using 1200 two-cell embryos. We do not know whether this indicates a transcriptional block of the endo A gene at this stage or if there are not enough endo A transcripts in these 2400 cells to be detected. However, low levels of correctly initiated endo A transcripts have been found in eight-cell morulae. The transition from the eight-cell morula to the blastocyst stage is characterized by a 10-fold increase in the level of endo A mRNA. This parallels a similar increase in the amount of the protein (10). Immunocytochemical studies have shown that endo A is expressed in the trophectoderm of the mouse blastocyst but not in the inner cell mass (5, 10). *In situ* hybridization experiments show that endo A mRNAs are also mainly found in the trophectoderm. In dissected 7.5-day embryos, the presence of endo A mRNA is restricted to the endoderm and the amnion, the only tissues in which the protein is also found.

Since both endo A protein and mRNA are present only in the trophectoderm, and later only in the endoderm and the amnion, it is likely that the expression of the endo A gene is regulated at the transcriptionnal level during the first differentiations of the mouse embryo. As early as the blastocyst stage, the expression of the endo A gene, which occurs from the eight-cell stage, is therefore regulated with a tissue-specific pattern. Since immunocytochemical studies did not allow detection of endo A in eight-cell morulae, it is not known whether or not endo A is expressed in all blastomeres or only in a few of them. *In situ* hybridization studies should provide an answer to this point.

Transition from the eight-cell morula to the blastocyst is the first morphological event of mammalian embryogenesis. Thus, expression of the endo A gene during blastocyst formation could be directly regulated by topological signals. If this is the case, analysis of the regulation of the transcription of the endo A gene provides an insight into molecular events related to a simple morphogenetical phenomenon.

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