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A labile inhibitor blocks endo A gene transcription in murine undifferentiated embryonal carcinoma cells

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ABSTRACT

The endo A gene encoding for an intermediate filament protein, a cytokeratin is usually expressed in epithelial cells. The regulation of this gene, probed by using cycloheximide, an inhibitor of protein synthesis was studied in various cell lines. The lines explored were undifferentiated embryonal carcinoma PCC4 cells which normally do not express endo A gene, PCC4 cells cultivated permanently at 31° C (PCC4-31), which are epithelial-like cells derived by differentiation from PCC4 cells, but which do express endo A gene, TDM1 cells, an epithelial teratocarcinoma cell line, and 3T6 mouse fibroblasts. Treatment of undifferentiated PCC4 cells by cycloheximide led to transcriptional induction of the endo A gene, and the same effect was observed after this treatment in PCC4-31 cells. By contrast, cycloheximide did not induce endo A gene expression in 3T6 cells, and reduced the transcriptional activity of this gene in TDM1 cells. We conclude that a labile inhibitor (or several) blocks endo A gene expression in undifferentiated PCC4 cells. We suggest that in these cells, the expression of the endo A gene is regulated both positively and negatively, possibly by a cellular E1A-like activity, as we previously demonstrated it for Py virus (C. Crémisi and C. Babinet, 1986. J. Virol. 59: 761-763). We further suggest that negative regulatory factors involved in this regulation are absent in TDM cells and reduced in PCC4-31 cells.

INTRODUCTION

Mouse embryonal carcinoma (EC) cell lines, derived from the stem cells of teratocarcinomas, provide an attractive in vitro model system for studying the regulation of gene expression during mouse early embryogenesis (1). EC cells have many features in common with the cells of preimplantation embryos (1, 2), for instance, they are undifferentiated and pluripotential. In vitro differentiation of EC cells can be triggered either by particular culture conditions (1, 3, 4) or by treatment with various drugs (5-9), and a variety of tissue types similar to those normally found in early embryos can thus be obtained.

During the last few years, the regulation of several cellular genes, which are silent in EC cells but expressed upon differentiation, has been intensively studied. Their expression is regulated mostly at the transcriptional level (for review 10, 11). Despite the progress made in the understanding of cellular gene expression upon EC cell differentiation, the precise mechanisms involved in the switching of these genes are not yet known.

However, studies of the blocking of the expression in EC cells of viruses, such as Polyoma (Py), Simian 40 (SV40) and Moloney murine leukemia virus (MoMuLV), have provided some insight into the mechanism(s) implicated in this switching (for review, see 10, 12). The absence

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of viral gene expression in EC cells has been found to be due, at least in part, to a functional deficiency of the viral enhancer (13, 14). We observed recently that transient treatment of PCC4 cells with an inhibitor of protein synthesis induced Py T antigen expression, suggesting that one or several labile repressor molecules might be partly responsible for the failure of Py DNA to be expressed in EC cells (15).

Other recent studies using viral DNA-mediated gene transfer also led to the conclusion that EC cells might contain negative regulatory factors acting on MoMuLV and SV40 enhancers (16). In addition, we and other authors observed that the growth of PCC4 cells at low temperature allows Py virus expression (4, 17). We further showed that at 31° C, PCC4 cells differentiated into epithelial-like cells and expressed a large amount of cytokeratin endo A (4). It is known that the endo A gene, which encodes an intermediate filament protein, a cytokeratin is not expressed at appreciable levels in EC cells, except in a few spontaneously differentiated cells, but is expressed when these cells treated by retinoic acid differentiate into epithelial-like cells (18, 19, 20).

As one or several proteins negatively regulate viral genes in EC cells, it is reasonable to assume that these protein(s) might also be involved in regulating certain cellular genes. As we previously suggested, the differentiation of PCC4 cells at 31° C is probably triggered, at least in part, by the derepression of certain cellular genes (15). In the present work, we therefore examined in detail the regulation of endo A gene expression in PCC4 cell lines cultured either at 37° C or at 31° C, in the presence of an inhibitor of protein synthesis. We also tested two other differentiated cell lines : TDM1, an epithelial teratocarcinoma cell line (3) and 3T6 fibroblast cells. Our results show that the expression of this gene can be induced at the transcriptional level in PCC4 cells by cycloheximide, suggesting that the blocking of its expression in EC cells may be partly due to a labile inhibitor.

EXPERIMENTAL PROCEDURES

Cells

The PCC4-aza-1 line is derived from the transplantable mouse teratocarcinoma OTT6050 and was selected for its resistance to azaguanine (3). When injected into strain 129 mice, these cells give rise to trigerminal teratocarcinoma. They grow in vitro as undifferentiated stem cells. To induce their differentiation into primitive epithelial cells, PCC4-aza-1 cells were grown at 31° C as we previously described (4). TDM1 is a trophoblastoma cell line derived from a teratocarcinoma (3). Cell cultures were treated with cycloheximide at a concentration of 5 µg/ml for 5 hours, and mRNA or nuclei were prepared immediately after this treatment. We observed that these conditions inhibit protein synthesis more than 95%, as measured by the decrease in the incorporation into protein of radioactively labelled amino-acid. Under these conditions, little cell death was observed.

RNA preparation

Total cytoplasmic RNA was prepared from cultured cell lines, as described by Maniatis et al. (21).

S1 mapping analysis of RNA

S1 mapping was performed as described by Duprey et al. (18). The probes, subcloned into m13mP8, were labelled by primer extension so as to attain a high specific activity. The endo A probe, which is a 345 bp fragment, encompassing the 5' end of the a 1 gene, has been described in detail (22). Upon S1 digestion of RNA-DNA hybrids, two fragments are protected : a major fragment of 130 nucleotide long and a minor fragment of 153 nucleotide long (19).

The Beta 2 microglobulin probe, a 200 bp fragment derived from the 3' end of Beta 2 microglobulin cDNA, has been described in detail (23).

The H-2k^b probe contains sequences derived from the first intron and the second exon of the H-2k^b gene (24). Upon S1 digestion, a 270 b fragment containing sequences derived from the second exon is protected.

Nuclear run off experiments

Nuclei were isolated from cultured cells as described by Groudine et al. (25), except that NP40 was not added to the lysis buffer and that the cells were disrupted in a dounce homogenizer by 10 to 20 strokes of a B pestle. About 10^8 cells were used per experiment. Nuclear run off experiments were performed as described (25, 26) except that a^{32} PUTP with a specific activity of 3000 Ci/mmole (Amersham) was used. Four µg of each of the following DNA fragments : a 1120 bp PSTI fragment derived from a mouse ß actin cDNA (a gift from M. Buckingham), a mixture of two BamHI-EcoRI fragments of 2000 bp and 2150 bp derived from the a1 endo A gene and PBR DNA linearized at PST site were loaded on a agarose gel and blotted on nitrocellulose filter. The filters were then hybridized to the runoff products for 48 hours as described by Groudine et al. (25). Equal amounts, measured by the number of counts per minute, of runoff products were added to each filter. The band corresponding to the a1 endo A gene appears to be broad because these two close fragments are present (see Fig. 1).

Densitometry

To quantitate the relative amounts of mRNA, the bands on the autoradiogram were scanned with a VERNON integrator-photometer and the surfaces under the peaks were measured. The densitometric values for the different bands were calculated by comparing bands of the same autoradiogram.

RESULTS

Effect of cycloheximide treatment on mRNA production

Analysis of the steady-state endo A mRNA in PCC4 cells by S1 nuclease mapping showed, as expected, that at 37° C, the amount of endo A transcripts in these cells was very small (Figure

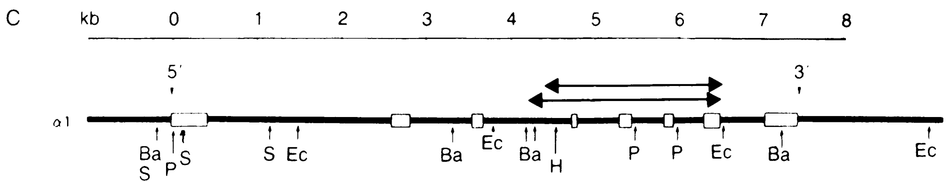

Fig. 1 : Structure of the endo A probe used for nuclear run off analysis. A schematic map of the endo A gene is shown. The DNA fragments used for nuclear run-off analysis are indicated by arrows. Ba, BamHI; S, Sma I; P, Pvu II; Ec, EcoRI; H, Hind III.

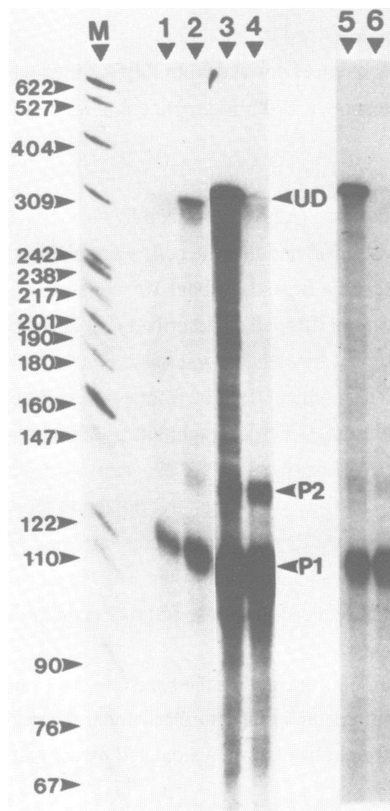

Fig. 2 : Nuclease S1 mapping of endo A mRNA in teratocarcinoma cells.The bands corresponding to the undigested probe (UD) and to the major (P1) and minor (P2) initiation sites are indicated by arrows (see Material and Methods). Illustrated is the hybridization of 30 μg of total cytoplasmic RNA extracted from lane 1-PCC4 cells, lane 2-PCC4 cells treated with cycloheximide, lane 3-PCC4-31 cells, lane 4-PCC4-31 cells treated with cycloheximide. M-markers. ^{32}P labelled HpaII-digested pBR322 DNA was used as a size marker. Autoradiograms were exposed for 15 hours at -70° C. Lane 5 and lane 6 show the same RNAs as in lane 3 and 4 except that the autoradiogram was exposed for 4 hours.

TABLE 1 : Quantitative analysis of endo A gene expression in PCC4 cells under different conditions

Cell type	PCC4	PCC4 + cycloheximide	PCC4-31	PCC4-31 + cycloheximide
S1 nuclease analysis	1	3	30	45
Runoff transcription analysis	1	3 to 4	5	25 to 30

The relative amount of RNA was determined by densitometry scanning.

2, lane 1). In fact, the endo A transcripts came from the few differentiated cells (less than 1 %) present in the population. We were able to test it by indirect immunofluorescence staining using an antibody to keratin filament (data not shown). To see whether the inhibition of protein synthesis would induce endo A gene expression as it induces Py expression (15), cells were treated with cycloheximide. Following this treatment, a three to fourfold increase was observed in the steady-state mRNA from this gene (Figure 2, lane 2 and Table 1).

At 31° C, the PCC4 cells differentiate spontaneously into epithelial-like cells and synthesize large amounts of endo A mRNA and cytokeratin protein (4). We found that the amount of endo A mRNA measured by densitometry of S1 analysis was at least 30 fold greater than that observed in cells cultured at 37° C (Figure 2, lane 3 and Table 1). When PCC4-31 cells were treated with cycloheximide, the amount of endo A mRNA was greater than that in untreated cells, but only by a factor of 1.4 (Figure 2, lane 4 and Table 1). All the transcripts we detected in PCC4 cells were correctly initiated, whatever the culture conditions used.

To establish whether this induction of endo A gene expression by cycloheximide was specific to EC cells, we tested two other differentiated cell lines . The first one, an epithelial teratocarcinoma cell line, called TDM1, is a trophectoderm-like derivative which synthesizes large amounts of endo A mRNA (19, and Figure 3, panel A, lane 1). The amount of endo A mRNA in these cells is about twofold higher than that in PCC4-31 cells.Treatment of TDM1 cells with cycloheximide not only failed to increase the amount of this RNA (Figure 3, panel A, lane 2), but slightly reduced it. The second differentiated cell line used consisted of fibroblast 3T6 cells, which do not express endo A at all. No endo A mRNA was detected in these cells, even after prolonged exposure of the autoradiogram of S1 analysis. Application of the same cycloheximide treatment to these cells did not induce endo A mRNA synthesis (data not shown).

In order to ascertain whether this induction of gene expression was limited to the endo A gene, we tested, under the same conditions, the H-2K^{b} and ß2-microglobulin genes, which code for histocompatibility antigens. Neither gene is expressed in EC cells, but both are

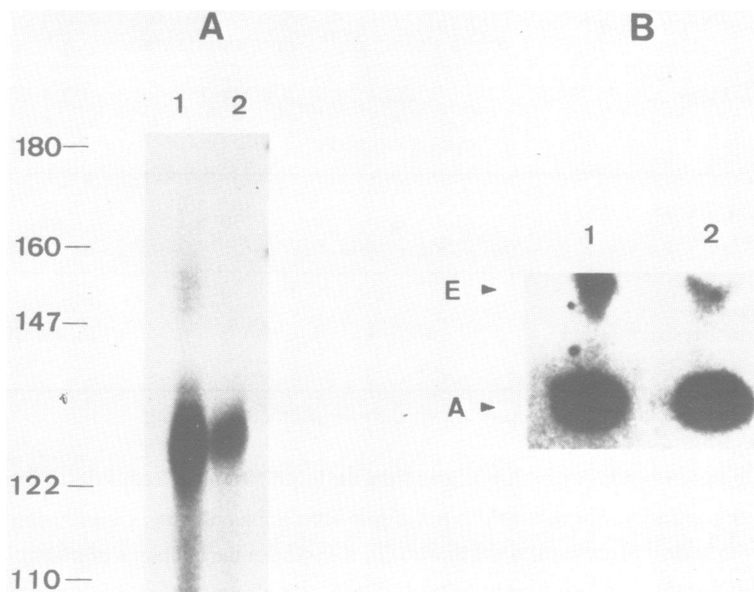

Fig. 3 : Effect of cycloheximide on endo A gene expression in TDM1 cells.Panel A : Nuclease S1 mapping of 30 µg cytoplasmic RNA (see Fig. 2 for details). Extracted from : (1) TDM1 cells, (2) TDM1 cells treated with cycloheximide. Exposure was 12 hours at - 70° C. Panel B : Runoff transcriptional analysis of endo A (E) and ß actin (A) genes in (1) TDM1 cells, (2) TDM1 cells treated with cycloheximide. Exposure was 4 days at -70° C.

coinduced upon differentiation (27). Here they were very slightly induced at low temperature (Figures 4 and 5). In most of the present experiments, we did not observe any induction of the expression of these genes after cycloheximide treatment at 37° C or 31° C, but occasionally detected small induction at both temperatures (Figure 4 and 5).

Transcriptional activity of endo A gene

Nuclear run off experiments were performed to establish whether or not the increase in endo A gene expression after cycloheximide treatment occurred at the transcriptional level. As previously reported, no new initiation occurs during the nuclear assay, and consequently polymerase density along specific genes can be determined (25). We found that endo A transcription in PCC4 cells increased three-to fourfold after cycloheximide treatment (Figure 6, lane 2 and Table 1). In PCC4-31 cells, before protein synthesis was inhibited, transcription was fivefold greater than in PCC4 cells. After cycloheximide treatment of PCC4-31 cells, endo A transcription was yet sixfold greater, i.e. about 30 times greater than in untreated PCC4 cells (Figure 6, lanes 3 and 4 and Table 1). The band in Fig. 6 corresponding to the endo A gene is broad, because we used two DNA fragments differing in length at the 5' end by 150 base pairs (see Materials and Methods for precisions). By constrast, the transcriptional activity of the endo

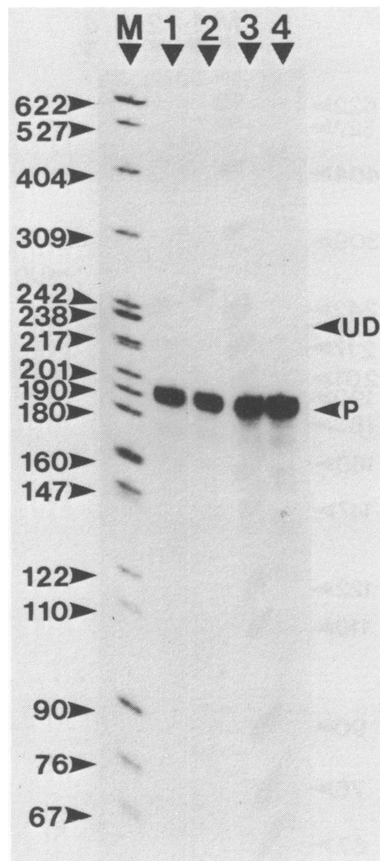

Fig. 4 : Nuclease S1 mapping of Beta 2 microglobulin mRNA in PCC4 cells.The bands corresponding to the undigested probe (UD) and the protected fragment (P) are indicated by arrows. Illustrated is the hybridization of 30 µg of total cytoplasmic RNA extracted from lane 1-PCC4 cells, lane 2-PCC4 cells treated with cycloheximide, lane 3-PCC4 31 cells, lane 4-PCC4 31 cells treated with cycloheximide. M : markers. Exposure was 48 hours at - 70° C.

A gene was decreased in TDM1 cells by cycloheximide treatment (Figure 3, panel B). In all these experiments, the transcription of the cellular ß actin gene, was included as a control. The transcription of this gene is very high and subject to little, if any, variation during EC cell differentiation (26) and appears as such in our study (Fig. 6). Plasmid DNA was included as a negative control and shows that the background level of hybridization is low (Fig. 6).

DISCUSSION

The experiments presented here, suggest that the endo A gene is negatively regulated in undifferentiated mouse embryonal carcinoma PCC4 cells. In these cells, the endo A gene is

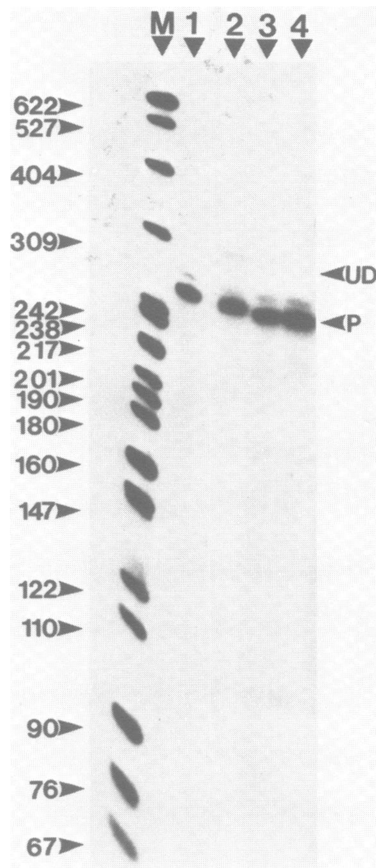

Fig. 5 : Nuclease S1 mapping of H2kb mRNA in PCC4 cells.The bands corresponding to the undigested probe (UD) and the protected fragment (P) are indicated by arrows. Illustrated is the hybridization of 50 µg of total cytoplasmic RNA extracted from lane 1-PCC4 cells, lane 2-PCC4 cells treated with cycloheximide lane 3-PCC4 31 cells, lane 4-PCC4 31 cells treated with cycloheximide. M : markers. Exposure was 48 hours at - 70° C.

normally not expressed; this block can be overcome by a brief treatment with an inhibitor of protein synthesis. This result suggests that the inhibitory factors preventing endo A gene transcription are short-lived proteins. Such labile inhibitors could block transcription directly by binding the endo A gene or by stabilizing the interaction of other negative regulatory factors with the endo A gene. Alternatively, the inhibitors could prevent endo A gene transcription indirectly, for example, by decreasing the level of cellular factors required for endo A transcription. Although it is not possible to distinguish between these two alternatives at present, indirect arguments discussed below, based on the comparative analysis of Py and endo A gene expression, favor the first alternative.

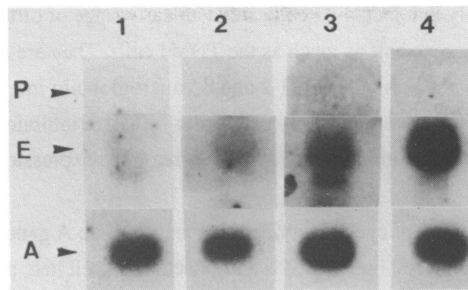

Fig. 6 : Runoff transcriptional analysis of endo A (E) and B actin (A) genes in PCC4 cells.Lane 1-PCC4 cells, lane 2-PCC4 cells treated with cycloheximide, lane 3-PCC4 31 cells, lane 4-PCC4 31 cells treated with cycloheximide. For endo A (E) and PBR (P) autoradiograms were exposed at - 70° C for 7 days and for B actin (A) for 20 hours.

Since in PCC4 cells, cycloheximide treatment induces transcription and increases the amount of endo A mRNA to the same extent, the induction of endo A expression in this case is only due to a transcriptional event.

That one can observe endo A transcription in PCC4 cells after cycloheximide treatment also suggests that positive trans-acting factors are already present. Possibly only a small amount of these factors is present prior to differentiation ; more may be produced upon differentiation as it occurs for the expression of Py and SV40 viruses in EC cells (15, 28).

We would like to mention that the increase transcription in treated PCC4 cells is due to increase transcription in the stem cells and not in the few differentiated cells preexisting in PCC4 population, since we observed by immunofluorescence staining using antibody to keratin filament, more stained cells after reversing the effect of cycloheximide treatment (data not shown).

At 31° C, the PCC4 cells differentiate into epithelial-like cells and synthesize large amounts of endo A mRNA (6, Fig. 2). In this case, the increase of mRNA by a factor of 30 is caused, in part, by an increase of the transcription rate by a factor of 5 and also by a post-transcriptional component, which increases the mRNA by a factor of 6 (Table 1). Similar observations were reported for the gene of collagen type IV after F9 EC cell differentiation (29).

Cycloheximide treatment of PCC4-31 cells still increases the amount of endo A mRNA in these cells. This increase occurs essentially at the transcriptional level by five-to sixfold, which is not reflected to such a level in the accumulated mRNA (Table 1). The effect of cycloheximide on the transcription in PCC4-31 cells can be explained by a reduction of labile inhibitors at that temperature, which would therefore facilitate the action of the drug. PCC4-31 cells might also have positive trans-acting regulatory factors in greater amount than in PCC4 cells. Whatever this amount, at 31° C, the activity of the negative regulatory factors is likely to be dominant over that of the positive one, because if not, cycloheximide would have reduced the amount of endo A mRNA transcripts as it did in TDM1 cells (discussed below).

We have shown recently that PCC4-31 cells are at an early stage of differentiation (4). These cells transcribe the endo A gene half as much as the TDM1 cells. They are still resistant to SV40 infection (4, 17) and only small amounts of H-2 and ß2 microglobulin transcripts are synthesized (4). This might explain why these cells still synthesize inhibitors, though in small amounts; consequently, cycloheximide is still able to increase the expression of the endo A gene in PCC4-31 cells.

We next analyzed the effect of cycloheximide treatment on endo A gene expression in another differentiated teratocarcinoma cell line ; TDM1, a trophectoderm cell line, producing large amounts of endo A proteins (17,18). The transcription rate of the endo A gene is reduced by cycloheximide treatment. This result shows that there is no negative regulatory factor in these cells and rather suggests the presence of positive regulatory trans-acting factors, the amounts of which are reduced by protein synthesis inhibitor(s). However, one cannot totally exclude the possibility that negative regulatory factors are present in these cells in small amounts.

We also tested another differentiated cell line, the fibroblast 3T6, which does not express the endo A gene at all. Cycloheximide treatment of these cells is not sufficient to induce endo A gene expression, suggesting that positive regulatory trans-acting factors are reduced in 3T6 cells. Whether or not inhibitors are present in these cells cannot be determined from our approach ; nevertheless, it is not impossible that these cells synthesize specific inhibitor(s) that are absent in EC cells.

From these results, we propose that the regulation of the endo A gene expression in undifferentiated EC cells, which are committed to expressing the endo A gene is under the control of positive and negative regulatory trans-acting factors as may occur in the first embryonic stages. The negative regulatory factors may prevent transcription by binding to the gene. During and after differentiation, in epithelial cells, i.e. expressing cells, the relative amount of these antagonistic factors would reverse, that is, the negative one would decrease progressively to zero as happens in TDM1 cells, while the positive one would increase. When the cells differentiate into cell types other than epithelial cells, the synthesis of positive trans-acting factors would be reduced and possibly, this effect might even be reinforced by the synthesis of new specific inhibitors absent in EC cells and the gene might remain definitively in an inactive domain of chromatin.

We would like to point out that the progressive decrease and increase of these factors is well illustrated by what is observed with the epithelial cell lines PCC4-31 and TDM1, which are not at the same stage of differentiation and which synthesize different amounts of endo A transcripts. The situation is similar during mouse embryogenesis, in which endo A transcripts start to be detectable at eight-cell stage but increase in large amounts in the trophectoderm layer of the blastocyst stage (19).

It is interesting to note that Py is negatively regulated in PCC4 cells, and that in PCC4-31 cells, Polyoma T antigen expression is enhanced by cycloheximide treatment (15), similarly to that of the endo A gene. Py expression, like that of the endo A gene, is a very early marker, as

both genes start to be expressed in the earliest differentiated cells, i.e. the trophectoderm layer of the blastocyst stage (19, 12). It is of interest to note that the expression of early markers, like endo A and Py, is more easily triggered and has a different regulation that of H-2 and β_2 microglobulin genes, which switch on later during development (23).

We propose that endo A gene and Py virus have negative regulatory trans-acting factors in common in PCC4 cells. In this regard, we find in the 5' untranslated region of the first exon, on the non coding strand of the endo A gene, DNA sequences homologous, to the E1A core enhancer and to the element A of Py enhancer, which is not functional in undifferentiated EC cells (see 13, 30).

PY-A-CORE	G	C	A	G	-	G	A	A	G
ENDO A	G	G	A	G	C	G	A	A	G
	+ 69								+ 61

On the other hand, some of the positive regulatory factors implicated in the regulation of these two genes should be different, because in 3T6 cells Py virus to the opposite to endo A gene is able to be expressed.

Using assays of transient expression, it was shown that Py and SV40 enhancer can be negatively regulated in Hela cells by E1A protein (31, 32). It is of interest to note that EC cells contain a cellular E1A-like activity (33) and that, as already mentioned, Py expression is negatively regulated in PCC4 cells, possibly by this cellular E1A-like activity. Taking all the data mentioned above into account, we propose that the endo A gene is negatively regulated in undifferentiated EC cells by a cellular factor with E1A-like activity. This cellular E1A-like activity probably has a pleiotropic effect on several different genes (33). It has been shown that the viral E1A proteins can act positively or negatively in regulating the expression of numerous viral and cellular genes ; Py and SV40 early genes (31, 32), an heat shock gene (34), an human ß-tubulin gene (35), a mouse MHC H-2K gene (36, 37) and an immunoglobulin gene (38). Furthermore, many regulated enhancer elements of viral and cellular genes were found to have homologous sequences in common (for review see 13, 31). Negative regulation is probably a general mechanism for controlling gene expression (for ref. see 31, 39). The expression of a gene may result from a subtle combination of specific and non-specific factors acting positively or negatively.

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