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Cell type-specific transcriptional enhancement *in vitro* requires the presence of *trans*-acting factors

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Cell-specific transcriptional enhancement was observed, depending on the enhancer sequences, using nuclear extracts prepared from B-cells, T-cells and HeLa cells. SV40 enhancer stimulated *in vitro* transcription up to 15-fold in all three cell extracts, whereas transcriptional potentiation *in vitro* by IgCμ and LPV enhancers was only seen in B- and T-cell extracts. Thus, the cell type specificity seen *in vivo* can be reproduced *in vitro*. The transcriptional enhancement requires the presence of enhancer sequences in *cis* and also of a common factor interacting in *trans* with all three enhancer sequences. Interestingly, first experiments indicate the additional presence of cellular factors in T-cell and most prominently in HeLa cell extracts which can reduce the enhancer activity of Cμ and LPV.

Key words: trans-acting factors/transcriptional enhancement/cell type specificity

Introduction

Enhancers represent a class of controlling elements that can increase transcriptional activity of many eucaryotic promoters relatively independently of distance and orientation with respect to the coding region (for review, see Khoury and Gruss, 1983). Although first discovered in tumor viruses (Benoist and Chambon, 1981; Gruss *et al.*, 1981), similar elements seem to be present in many eucaryotic genes such as immunoglobulin (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Queen and Baltimore, 1983), insulin, chymotrypsin, amylase (Walker *et al.*, 1983; Rutter, personal communication), elastase I (Swift *et al.*, 1984) and metallothionin (Séguin *et al.*, 1984; Karin *et al.*, 1985). Strikingly, most cellular enhancers (see above) and also some viral enhancers (Sodroski *et al.*, 1984; Mosthaf *et al.*, 1985) exhibit their activity in a highly cell-type specific manner. With the exception of a short stretch of nucleotides (Weiher *et al.*, 1983; Hen *et al.*, 1983), different enhancer elements bear no extensive sequence homology; these sequence differences could serve as an explanation for the obvious cell-specific activity profile. Additionally, however, cellular factors are required to mediate enhancer function *in vivo* (Schöler and Gruss, 1984) and also *in vitro* (Wildeman *et al.*, 1984; Sassone-Corsi *et al.*, 1985). It seems, therefore, that the cell-specific function of some enhancers is dependent on *trans*-interacting cellular factors present only in certain cells and tissues.

To study the cell-specific effects in molecular detail, appropriate *in vitro* systems are an absolute prerequisite. Recently, cell-free extracts were prepared from HeLa cells that largely mimic enhancer activity *in vitro* (Sassone-Corsi *et al.*, 1984; Sergeant *et al.*, 1984). In these experiments, the enhancer of SV40 proved to stimulate transcriptional activity severalfold. However, in contrast to the SV40 enhancer sequences that are promiscuously active in almost all cells studied (Mosthaf *et al.*, 1985), the examination of cell-specific transcriptional enhancement requires the utilization of a highly cell- or tissue-specific enhancer in conjunction with the development of an appropriate cell-free extract. Toward this end, we used enhancers active in cells of the lymphoid system and, as a control, SV40 enhancer sequences. Cell-free extracts were prepared from human B and T cells and, as a control, from HeLa cells. Interestingly, enhancers active in lymphoid cells stimulated transcription *in vitro* only in extracts from B and T cells and not from HeLa cells. Competition experiments *in vitro* demonstrated that this activity is due to the presence of cellular factors that are required for enhancer activity.

Results

Experimental strategy: cell-specific enhancement in vitro

Cell-free systems allowing accurate and specific RNA polymerase II transcription of exogenous DNA templates have been described previously (Weil *et al.*, 1979; Manley *et al.*, 1980) and derive mostly from HeLa or KB cells growing in suspension. With these cell-free systems it has recently been demonstrated that the presence of SV40 enhancer sequences at or near the 5′ end of different promoters leads to a 5- to 10-fold increase in transcriptional activity of exogenous DNA (Sassone-Corsi *et al.*, 1984; Sergeant *et al.*, 1984). Although the majority of transcripts start at the correct position, several classes of RNA were transcribed from positions not recognized in *in vivo* experiments. The presence of these RNA classes, which most likely start unspecifically, can be greatly reduced if, instead of a whole cell extract, a nuclear extract is prepared (Dignam *et al.*, 1983; Wildeman *et al.*, 1984).

Initially we used such a nuclear extract prepared from HeLa cells to test for transcriptional potentiation of several different enhancer elements. All plasmid constructions used had been tested previously in *in vivo* experiments (Mosthaf *et al.*, 1985) and are outlined schematically in Figure 1B. In brief, four constructions were employed that all carry identical SV40 promoter sequences (21-bp repeats, TATA box) in front of a procaryotic gene (CAT) and upstream of it either one of three different enhancer elements (SV40, IgCμ, LPV) or none at all. Using this general set up, it is safe to assume that possible cell type-specific enhancement effects are due to the enhancer sequences and not to other control elements such as promoters. In our experiments, we used single-stranded DNA probes in a nuclease S1 assay in order to quantify the amount of transcription produced in the nuclear extracts from closed circular DNA templates (see Materials and methods for details). As shown in Figure 1A, a nuclear extract prepared from HeLa cells yields ~4- to 5-fold more transcripts when SV40 enhancer sequences are present on the plasmid. The position of the 5′ ends of the *in vitro* RNA is identical with the 5′ ends found *in vivo* (for review, see Tooze, 1981). No difference in the amount of RNA produced can be seen in the HeLa-

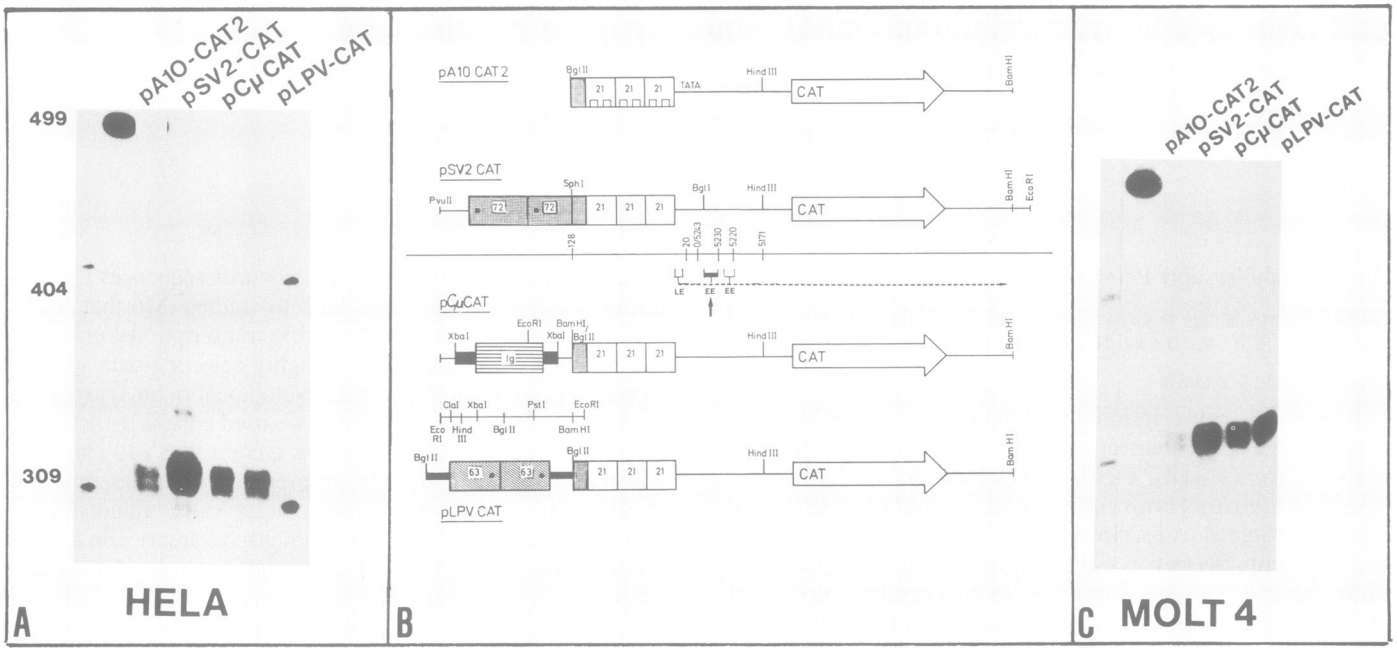

Fig. 1. **(A)** Effect of different enhancers on transcription from the SV40 early promoter with a nuclear extract of HeLa cells. Transcription of pA10-CAT2, pSV2-CAT, pCμ-CAT and pLPV-CAT and S1 analysis were carried out as described in Materials and methods. $MgCl_2$ and spermidine were added to 6 mM and 1 mM, respectively. The *Hpa*II length marker (M, 404, 309) and the SV40 S1 probe (499) are indicated. **(B)** Structural features of recombinants used for studying the *in vitro* effect of different enhancers on transcription from the SV40 early promoter. Each recombinant contains the SV40 promoter/origin region from *Sph*I (128) to *Hind*III (5171) fused to the CAT gene (Gorman *et al.*, 1982). In addition, they carry either one of the three different enhancer elements SV40, IgCμ, LPV with the exception of pA10-CAT2 (Mosthaf *et al.*, 1985). Nucleotide coordinates for the SV40 promoter/origin region follow the BBB system (Tooze, 1982). **(C)** Effect of different enhancers on transcription from the SV40 early promoter with a nuclear extract of Molt 4 cells. Transcription of pA10-CAT2, pSV2-CAT, pCμ-CAT and LPV-CAT and S1 analysis were as described in Materials and methods. $MgCl_2$ and spermidine were added to 6 mM and 2 mM, respectively. The *Hpa*II length marker (M, 404, 309) and the SV40 S1 probe (499) are indicated (left).

cell extract between a construction lacking enhancer sequences and constructions containing LPV or Cμ enhancer elements 5′ of the SV40 promoter. Thus enhancer elements such as LPV and Cμ, that are inactive in HeLa cells, are also inactive in nuclear extracts from HeLa cells. To see an *in vitro* potentiation effect from lymphoid-specific enhancers, we prepared a nuclear extract from BJA-B cells, a human EBV-negative Burkitt's lymphoma-derived cell line (Klein *et al.*, 1975). If Cμ enhancer sequences are present on the template transcription is stimulated (Figure 2). The degree of transcriptional stimulation, however, depends on the concentration of spermidine used in the extract (Figure 2). A drastic increase (up to 15-fold) can be observed if 2, 3 or 4 mM spermidine is present. Again, the 5′ ends of the RNA produced *in vitro* are at the expected positions.

Thus, nuclear extracts from HeLa cells and BJA-B cells can be used to reproduce the cell-specific enhancement effect *in vitro*. We subsequently prepared a nuclear extract from Molt 4, a human cell line derived from a patient with acute lymphoblastic leukemia of the T-cell type (Minowada *et al.*, 1972). As Figure 1C shows, considerably more RNA is produced from plasmid templates containing either SV40 (10-fold), Cμ (5-fold) or LPV (8-fold) enhancer sequences compared with pA10-CAT2, the enhancer-minus control plasmid. These data demonstrate that enhancer sequences with a cell-specific *in vivo* activity profile function *in vitro*, depending on the source of the cell extracts.

In vitro stimulation depends on enhancer sequences and requires their location in the vicinity of a promoter

To define unambiguously the sequences responsible for the enhancement effect and to rule out a hypothetical negative effect exerted on the enhancer-minus construction (pA10-CAT2), we analysed mutants carrying multiple point exchanges mainly in the 72-bp unit of the SV40 enhancer, which had been previously analysed *in vivo* (Weiher *et al.*, 1983). As Figure 3A shows the *in vivo* and *in vitro* results correlate well. Using a nuclear extract derived from Molt 4 cells we found that mutant #5 (pSV5-CAT; lane 3), which is extremely reduced in its activity in three independent *in vivo* assays (Weiher *et al.*, 1983), does not stimulate transcription *in vitro*, either. Also, mutant #18 (pSV18-CAT), which exhibits an intermediate phenotype *in vivo* (Weiher *et al.*, 1983) stimulates transcription *in vitro* to a lesser degree (Figure 3A, lane 2). These results establish that sequences within the 72-bp enhancer unit of SV40 are required for the *in vitro* potentiation effect.

Since enhancer sequences can exert their function independently of orientation and, according to the promoter used, relatively independently of distance with respect to the coding region (for review, see Khoury and Gruss, 1983), we were interested in determining whether these activity patterns could be reproduced *in vitro* using a Molt 4 extract and the cell-specific enhancers LPV and Cμ. Compared with pA10-CAT2 (Figure 3B, lane 1) both LPV enhancer (Figure 3B, lanes 2,4) and Cμ enhancer sequences (lanes 5,6) stimulate *in vitro* transcription regardless of orientation if positioned in the immediate vicinity of the SV40 promoter elements (21-bp, TATA box). On the other hand, if the LPV enhancer element is positioned ~2.5 kb away from the next available promoter no *in vitro* transcriptional stimulation can be observed (Figure 3B, lane 3), although the same recombinant is active in transient *in vivo* assays (Mosthaf *et al.*, 1985). Thus, in our *in vitro* experiments, as in some *in vivo* experiments, depending on the promoter used (Wasylyk *et al.*, 1984), a distance dependence of the enhancement effect is observed. Hav-

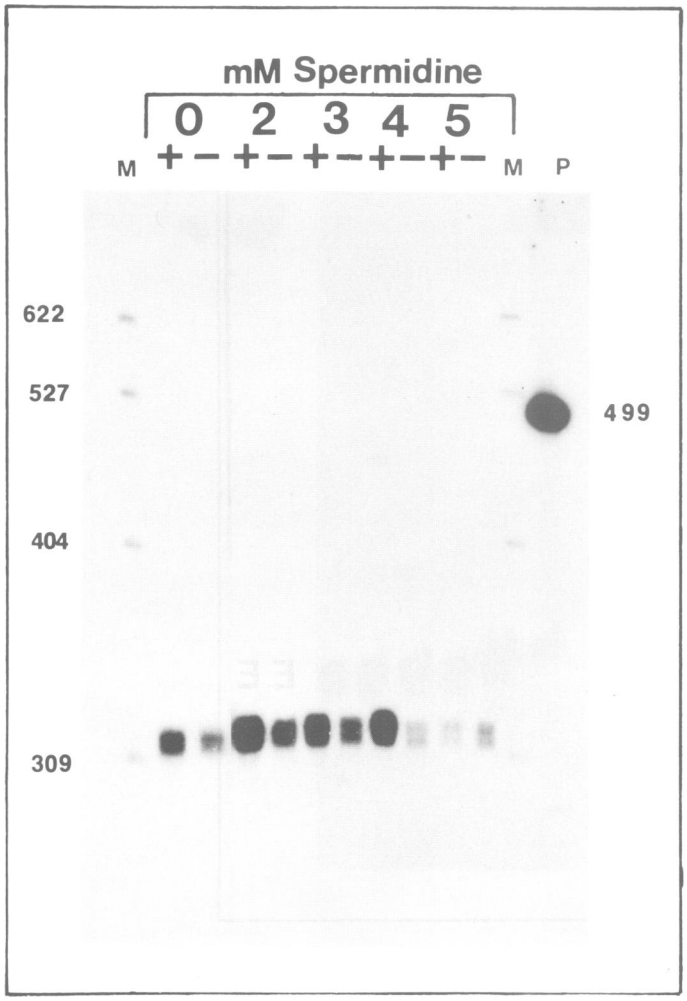

Fig. 2. Effect of sperimidine on transcription with or without the IgCμ enhancer in front of the SV40 early promoter in a nuclear extract of BJA-B cells. pCu-CAT (+) and pA10-CAT2 (−) were incubated at constant $MgCl_2$ and variable spermidine concentrations (above, as indicated) with BJA-B extract as described in Materials and methods. The *Hpa*II length marker (M) and the SV40 probe (P, 499) are indicated.

ing investigated characteristics of sequences required in *cis* for the enhancement effect *in vitro*, we next asked whether there is also a necessity for *trans* acting elements.

Specific interaction between enhancer sequences and cellular components in vitro

Competition assays had demonstrated the requirement of cellular factors for enhancer function *in vivo* (Schöler and Gruss, 1984), and using HeLa whole-cell extracts, *in vitro* (Wildeman *et al.*, 1984; Sassone-Corsi *et al.*, 1985). We were interested in determining whether cell-specific enhancer function is also exerted through interaction of cellular components. As an approach to this problem, we utilized nuclear extracts from Molt 4 cells for competition assays. Plasmid pCμ-CAT (Figure 4, bottom) was used as a template and either specific or non-specific competitor DNA was added prior to *in vitro* transcription. In all experiments, the total linear DNA concentration was kept constant by addition of pBR322 DNA, which therefore served as non-specific competitor. Specific competitor DNA was added (see Figure 4, b−d and e−g from left to right) in a 1:2.5, 1:5, or 1:10 molar ratio. As Figure 4 shows, increasing the concentration of Cμ enhancer sequences (1-kb *Xba*I fragment) results in a reduction of transcriptional activity *in vitro* (lanes e−g). Importantly, a plasmid carrying the α2-globin gene, which was used as an internal standard template in some of these experiments, was not reduced in its transcriptional activity (data not shown). This shows that competition involves enhancer-interacting molecules and not other, more general transcriptional factors, for example RNA polymerase II, also required for transcription of the α2-globin gene.

Interestingly, the reduction of transcriptional activity using the Cμ enhancer element as competitor decreased the transcription to a level normally seen with an enhancer-minus plasmid pA10-CAT2 (Figure 5A; compare c lanes with pA10-CAT2). Since this plasmid carries SV40 promoter elements (21-bp repeat, TATA box) and since previous *in vivo* experiments demonstrated that enhancer activity can be exerted only through natural or substitute promoters (Moreau *et al.*, 1981; Wasylyk *et al.*, 1983), we next attempted to compete for promoter factors required in *trans*. As demonstrated in Figure 4, lanes b−d, a fragment (*Sph*I-*Hind*III) which contains the SV40 promoter/origin region (Tooze, 1981) efficiently competes for transcriptional activity *in vitro*, thus showing a requirement of *trans* factors, possibly Sp1 (Dynan and Tjian, 1983), interacting with the SV40 promoter for exertion of enhancer activity.

To determine whether a common set of similar factors is required for the function of different enhancers in Molt 4 nuclear extracts, different templates were used in the *in vitro* competition assays. Using pLVP-CAT as template and the 1-kb *Xba*I Cμ enhancer fragment as competitor, a reduction of transcriptional activity could be observed by increasing the amount of competitor (data not shown). Similarly, when pSV2-CAT was used as template its activity could also be decreased by increasing the concentration of Cμ enhancer sequences (data not shown). Similar results were obtained in a complementary experiment in which a permuted version of the 72-bp repeat of SV40 (*Sph*I-cleaved monomer) was used and demonstrated reduction of transcriptional activity regardless of whether SV40 or Cμ enhancer sequences were present on the template (data not shown). Thus, at least one set of cellular factors present in Molt 4 cells can interact with SV40, Cμ and LPV enhancer sequences despite their greatly differing activity profiles.

Indications that negative trans interacting cellular factors are present in Molt 4 extracts

During the course of the competition experiments we attempted to delineate further the nucleotide sequences involved in the binding of cellular factors present in Molt 4 extracts. For this reason we used different restriction enzyme fragments of the mouse Cμ enhancer element as competitor (678-bp *Xba*I-*Eco*RI: fragment A, 305-bp *Xba*I-*Eco*RI: fragment B). As shown in Figure 5A (pCμCAT) the *Xba*I-*Eco*RI fragment A competes for Cμ enhancer activity. In parallel experiments a similar competition effect was also exerted on SV40 and LPV enhancer sequences (Figure 5A). Thus, a common, positively interacting *trans* factor may interact with sequences located in the 5′ domain of the Cμ enhancer-containing fragment (note that the transcriptional activity of the α2-globin template used as an internal standard remains unchanged). After increasing the amount of the smaller *Xba*I-*Eco*RI fragment B no decrease was observed (Figure 5B, C). In contrast, when pCμ-CAT and pLPV-CAT were used as templates, increasing the concentration of the *Xba*I-*Eco*RI fragment B resulted in a slight, but reproducible stimulation of transcription. Interestingly, when the highest level of competitor was used, the absolute amount of transcriptional activity reached or even exceeded the level of *in vitro* enhancement exerted by the SV40 enhancer ele-

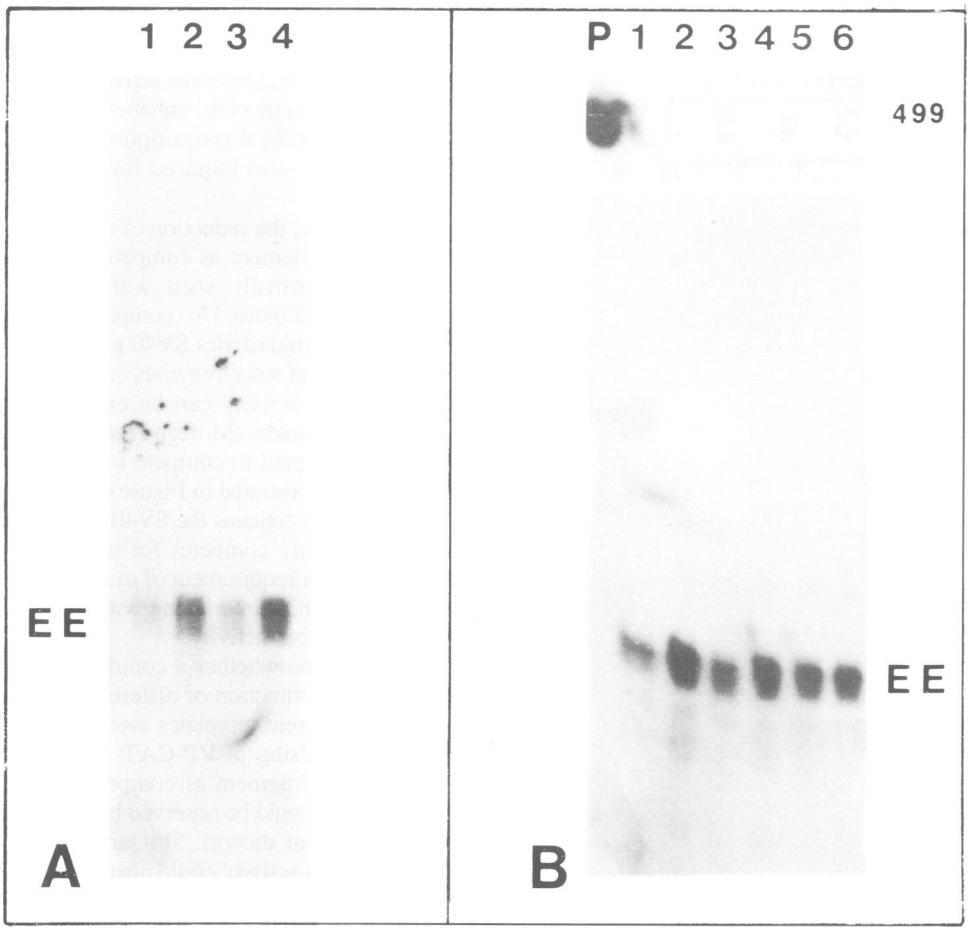

Fig. 3. **(A)** Effect of SV40 mutations on transcription from the SV40 early promoter in a Molt 4 extract. Transcription of **(1)** pA10-CAT2, **(2)** pSV18-CAT, **(3)** pSV5-CAT and **(4)** pSV2-CAT and S1 analysis were carried out as described in Materials and methods. $MgCl_2$ and spermidine were added to 6 mM and 1 mM, respectively. **(B)** Effect of distance and orientation of enhancers on transcription from the SV40 early promoter in a Molt 4 extract. Transcription of **(1)** pA10-CAT2, **(2)**, pLPV-CAT (5's), **(3)** pLPV-CAT (3's), **(4)** pLPV-CAT (5'a), **(5)** pCμ-CAT (5's) and **(6)** pCμ-CAT (5'a) and S1 analysis were carried out as described in Materials and methods. $MgCl_2$ and spermidine were added to 6 mM and 1 mM, respectively. 5' and 3' indicate the point of enhancer insertion corresponding to the gene; s and a refer to the sense and anti-sense orientation of the enhancers with respect to the coding region of the original gene. The SV40 probe (P, 499) is indicated.

ment (Figure 5B, C). No such stimulation, however, was observed either when the SV40 enhancer activity was competed by using the *Xba*I-*Eco*RI fragment B as competitor (Figure 5B, pSV2CAT) or when pA10-CAT2 was used as a template (data not shown). Thus, these results suggest the presence of cellular factors negatively interacting with sequences located on the small *Xba*I-*Eco*RI fragment B of the Cμ enhancer element.

If T-cells like Molt 4 harbor a certain amount of negatively interfering cellular factors, HeLa cells in which LPV and Cμ enhancers are completely inactive could have a different or a higher concentration of similar factors exerting this 'shut-down' effect. Therefore, to confirm the existence of negatively interacting factors, we analysed the transcriptional behavior of SV40, Cμ and LPV enhancers after mixing a HeLa cell extract (only the SV40 enhancer activates transcription) with a Molt 4 extract (all three enhancers stimulate transcription). As shown in Figure 6 (and Figure 1A) in a HeLa extract only SV40 enhancer sequences stimulate transcription *in vitro*, whereas in a Molt 4 extract SV40, Cμ and LPV enhancer elements stimulate *in vitro* transcription (Figure 6 and Figure 1C). Mixing HeLa and Molt 4 extracts in a 1:1 ratio results in a transcriptional pattern identical to the HeLa extract (Figure 6, center). Thus, the activity profile exerted in HeLa extracts is dominant over the activity exerted in Molt 4 extracts. Negatively interacting cellular factors again could be one explanation for this result.

Discussion

Eucaryotic transcriptional enhancers are unique in their mechanism of function because they act relatively independently of orientation and distance with respect to promoter sequences (for review, see Khoury and Gruss, 1983). Although the nucleotide sequences required in *cis* by at least some of the enhancers are well mapped (for review, see Gruss, 1985), little information is available concerning cellular factors required in *trans* to interact with enhancer elements (Schöler and Gruss, 1984; Wildeman *et al.*, 1984). A characterization of these cellular components seems to be a prerequisite for understanding the cell type specificity exerted by some enhancers (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Queen and Baltimore, 1983; Walker *et al.*, 1983; Swift *et al.*, 1984; Mosthaf *et al.*, 1985). However, detailed molecular analysis of the enhancer interacting molecules requires the development of appropriate *in vitro* systems. To study cell-specific enhancement *in vitro* we have adapted a protocol for the preparation of a nuclear extract from HeLa cells (Dignam *et al.*, 1983; Wildeman *et al.*, 1984) and prepared similar extracts from

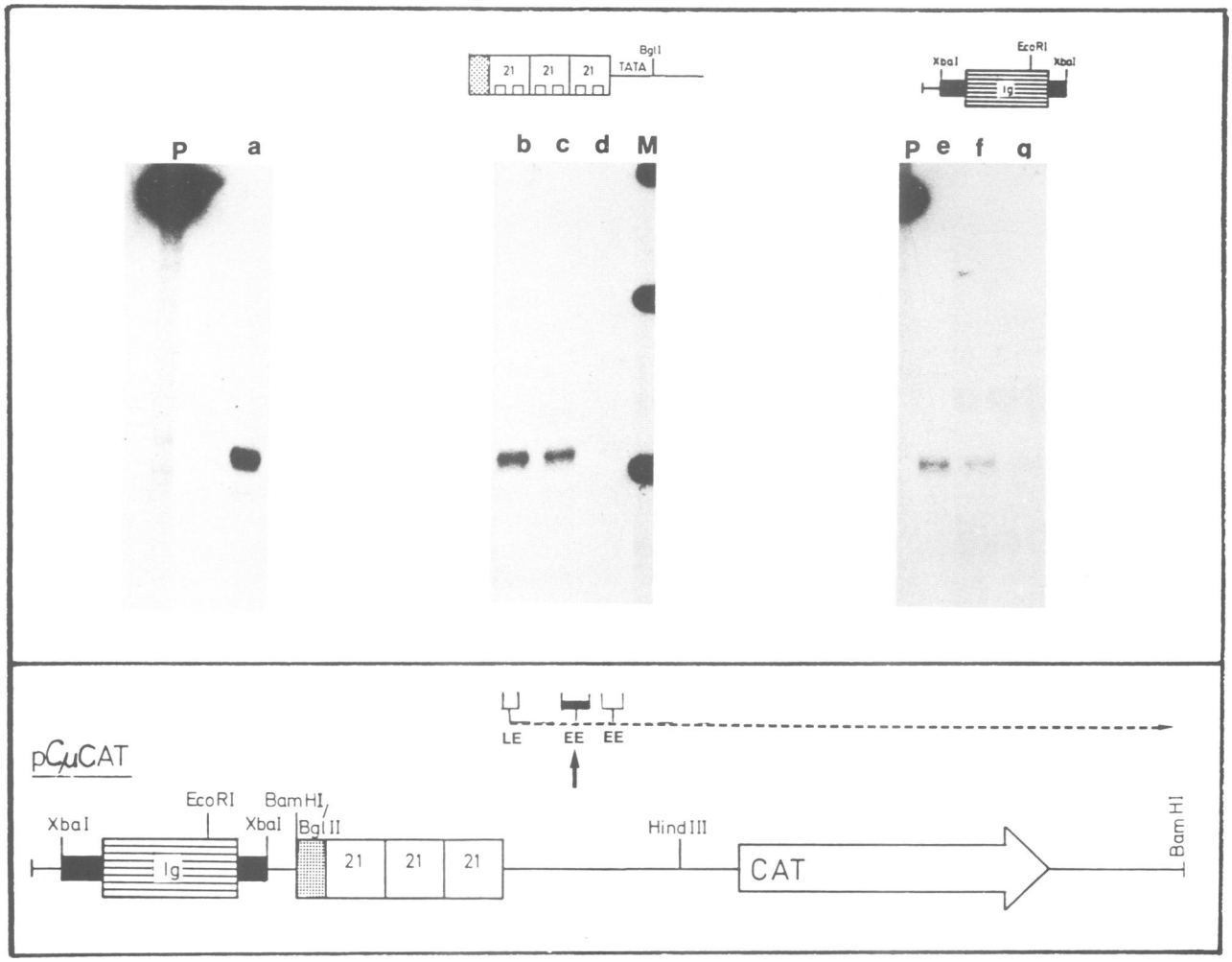

Fig. 4. Competition activity of the IgCμ enhancer and the SV40 early promoter on transcription of pCμCAT in a Molt 4 extract. 8 μg/ml of pCμCAT (bottom) was incubated in a Molt 4 extract with DNA fragments of pBR322 **(lane a)**, increasing concentrations of either the SV40 promoter/origin region [*Sph*I (128) to *Hind*III (5171)] **(lanes b–d)** or the IgCμ *Xba*I fragment **(lanes e–g)**. The molar ratios of competitor to test gene were 2.5:1 **(lanes b,e)**, 5:1 **(lanes c,f)** and 10:1 **(lanes d,g)**. The pBR322 fragments used in **lane a** were also taken to achieve the same amounts of linear DNA in each reaction. pUC8 was added to a final concentration of 40 μg/ml. Transcription and analysis of RNA were carried out as described in Materials and methods. $MgCl_2$ and spermidine were added to 6 mM and 1 mM, respectively. The *Hpa*II length marker (M: 527, 404 and 309) and the SV40 S1 probe (P, 499) are indicated.

human B- and T-cells. Our results indicate that DNA templates for polymerase II containing SV40 promoter elements (21-bp repeats, TATA box) downstream of either SV40, IgCμ, LPV or no enhancer sequences are specifically and accurately transcribed in all extracts tested. Using quantitative nuclease S1 analysis we found that the SV40 early-early (EE) start sites (Ghosh *et al.*, 1981; Benoist and Chambon, 1981) are used preferentially by all templates employed. These data are in good agreement with previous reports in which HeLa whole cell extracts were studied (Sassone-Corsi *et al.*, 1984).

We were most interested in determining whether different enhancers show a cell-specific activity profile depending on the origin of the nuclear extract. Interestingly, enhancers active in lymphoid cells *in vivo* such as Cμ, LPV and SV40 also stimulated transcription *in vitro* in B- and T-cell extracts and this enhancement effect was mediated by enhancer sequences. The degree of stimulation could be as great as 15-fold; however, certain variations were seen, even if several extracts were prepared from the same cell line. Leakage of some of the factors involved during the preparation of the extracts could be one explanation for these variations. Using a HeLa cell extract as control, we observed transcriptional stimulation only if the SV40 enhancer was present on the template; in this extract Cμ and LPV enhancer sequences remained functionally inactive, a result which is a reflection of the *in vivo* situation (Mosthaf *et al.*, 1985). Thus, cell-specific transcriptional enhancement can be reproduced *in vitro* depending on the enhancer and the source of the cell-free extract.

Not all enhancer characteristics observed *in vivo*, however, could be reproduced *in vitro*. Although both Cμ and LPV enhancer sequences show the transcriptional potentiation effect, regardless of their orientations, the location of the enhancer sequences on the template seems to be crucial for their functioning *in vitro*. Using the LPV enhancer, activity was only seen if the enhancer element was positioned in the immediate vicinity of the SV40 promoter and not if located ~3.9 kb 5′ or 1.8 kb 3′ from the SV40 promoter, suggesting a distance dependence for the *in vitro* enhancement effect. Corresponding data have been published analysing the function of SV40 enhancer elements in HeLa cell extracts (Sassone-Corsi *et al.*, 1984). Similarly, a distance dependence *in vivo* has been previously described for an enhancement of the conalbumin and SV40 promoter activity (Wasylyk *et al.*, 1984). The reason for this distance dependence remains to be determined.

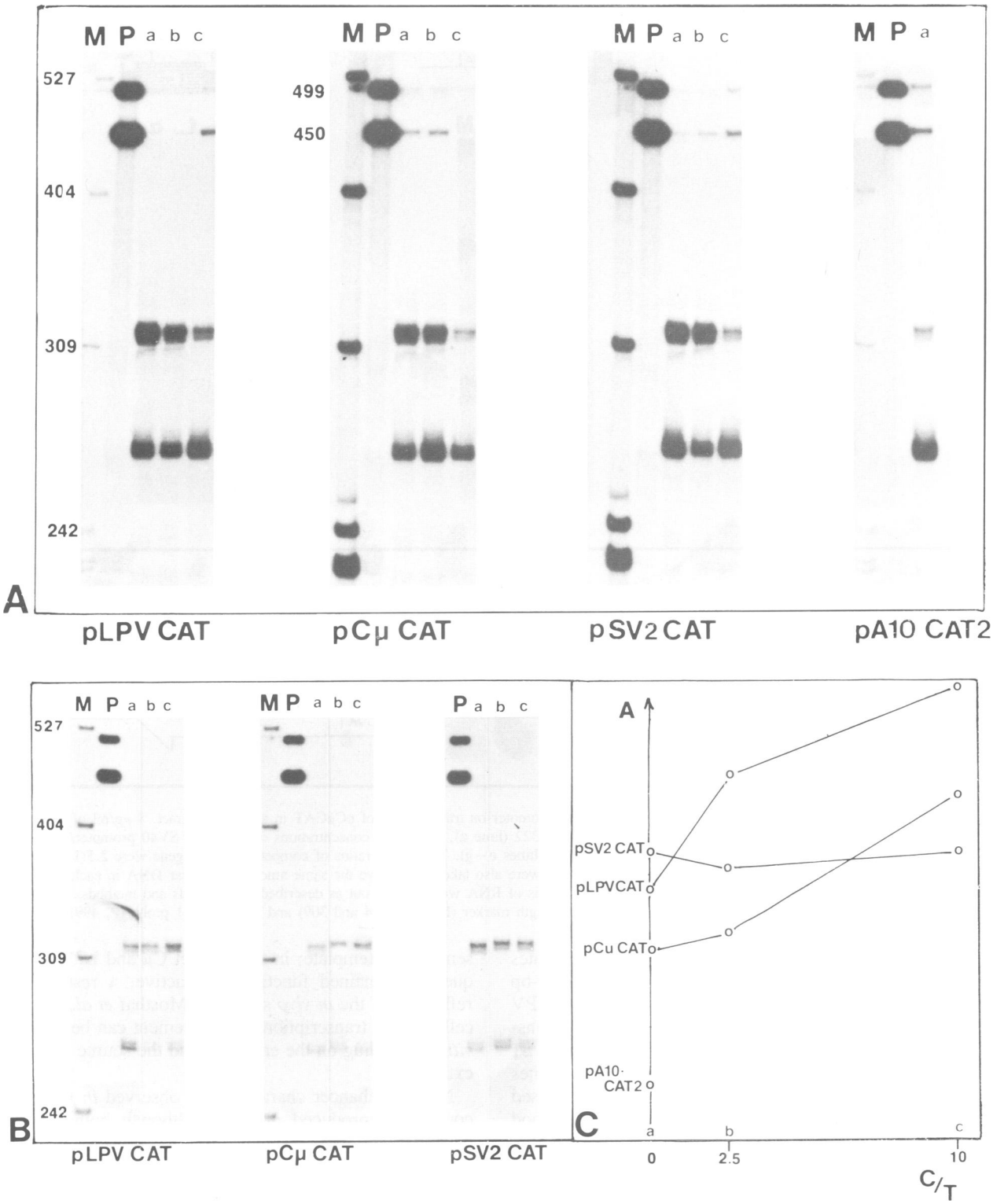

Fig. 5. Competition activity of subfragments of the IgCμ enhancer-containing *Xba*I fragment on different enhancers in a Molt 4 extract. 8 μg/ml pSV2-CAT, pLPV-CAT, pCμ-CAT and pA10-CAT2 (bottom, as indicated) were incubated with fragments of pBR322 DNA or subfragments of the IgCμ enhancer-containing *Xba*I fragment. In **A** the 678-bp *Xba*I-*Eco*RI fragment A, in **B** the 305-bp *Xba*I-*Eco*RI fragment B were used for competition; the molar ratios of competitor to test gene DNA were 2.5:1 **(lane b)** or 10:1 **(lane c)**. The pBR322 DNA fragments (of **lane a**) were also used to achieve the same amount of linear DNA in each reaction. 2.5 μg/ml of a plasmid carrying the human α2-globin gene was used as an internal standard. pUC8 was added to a final concentration of 40 μg/ml. Transcription and analysis of RNA were carried out as described in Materials and methods. $MgCl_2$ and spermidine were added to 6 mM and 4 mM, respectively. The pBR322 *Hpa*II length marker (M) and the mixed S1 probes (SV40: 499, 2-globin: 450) are indicated. The autoradiogram shown in **A** is the result of a 14 h exposure and the one in **B** was exposed for 3 days. **(C)** The levels of transcriptional activity (=A) were quantified densitometrically and plotted against different molar ratios of competitor to test gene DNA (C/T).

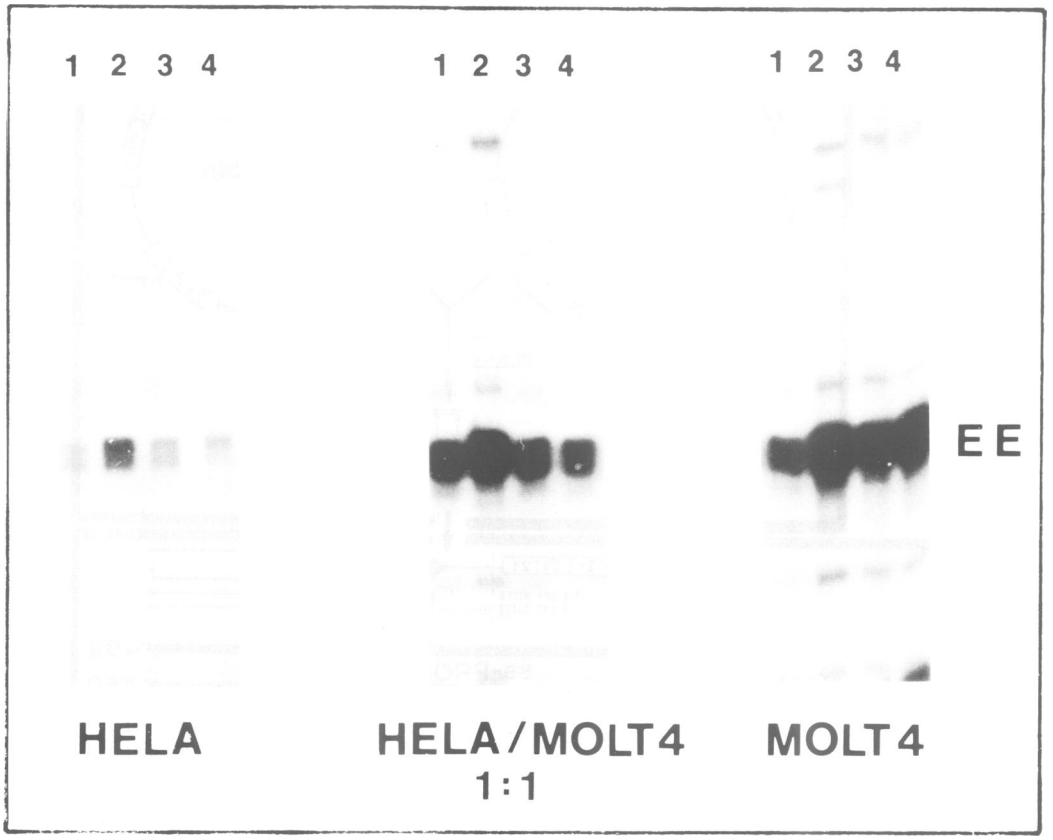

Fig. 6. Mixing of HeLa and Molt 4 extracts pA10-CAT2 **(1)**, pSV2-CAT **(2)**, pCμ-CAT **(3)** and pLPV-CAT **(4)** were transcribed with 6 mM $MgCl_2$ and 2 mM spermidine as described in Materials and methods. At the bottom of the figure: either HeLa (left), Molt 4 (right) or a mixture of both extracts (middle) are indicated.

With the help of transient expression systems we have previously determined the activity of SV40, Cμ and LPV enhancer sequences in a variety of cell lines (Mosthaf *et al.*, 1985). We demonstrated in these experiments that all three enhancers mediated transcriptional activities in BJA-B cells but only SV40 and LPV sequences stimulated transcription in Molt 4 cells as compared with the Cμ enhancer. Thus, we were surprised to discover that in nuclear extracts derived from Molt 4 cells transcriptional potentiation was seen even by the Cμ enhancer element although to a lesser degree than by SV40 and LPV. An explanation for this result could be provided by the competition experiments performed. Using all three enhancers with appropriate individual templates and purified fragments as competitors we demonstrated that a common set of factors is present in Molt 4 extracts that binds specifically to enhancer sequences and not to promoters such as α2-globin. In these experiments the Cμ enhancer sequences competed for the transcriptional potentiation effect mediated by SV40 and LPV *in vitro*. Thus even though the Cμ enhancer element seems to be inactive *in vivo* in Molt 4 cells and less active *in vitro* it nevertheless binds a set of seemingly common cellular factors, confirming previous results obtained in HeLa whole-cell extracts (Wildeman *et al.*, 1984; Sassone-Corsi *et al.*, 1985). To explain these data one could speculate that cell-specific expression involves both positive and negative regulatory events. The negative control could be exerted in all cells normally not responding to some cell-specific enhancers such as Cμ. The preparation of Molt 4 extracts might result in the loss of a certain amount of these putative negatively interacting molecules, thus allowing a certain degree of transcriptional stimulation to be seen mediated even by the Cμ enhancer.

To substantiate this hypothesis we attempted to map the sequences required for competition on the Cμ enhancer element. We analysed the effect of a 678-bp *Xba*I-*Eco*RI fragment which retains most of the *in vivo* activity (Banerji *et al.*, 1983). Using this fragment in an *in vitro* competition assay we found that it contains a region which can compete for common enhancer factors since increasing its concentration resulted in a decrease of transcriptional stimulation exerted by either the Cμ, SV40 or LPV enhancer. Curiously, a slight increase in transcriptional activity was observed when the small (305-bp) *Eco*RI-*Xba*I fragment was used as competitor for a template containing either the 1-kb Cμ or the LPV enhancer elements. No increase was observed when this fragment was employed in a competition assay using the SV40 enhancer-containing plasmid as a template. Thus, these results are compatible with the notion that a cellular factor binding to the 3′ portion of the Cμ enhancer represses the *in vitro* activity of this enhancer in Molt 4 cell-free extracts. The question, however, remains as to why the LPV enhancer element is also stimulated slightly by competition with fragment B. Further evidence for the presence of negatively acting factors was the dominance of the HeLa cell activity profile if mixed with appropriate Molt 4 extracts. Despite the presence of Molt 4 cellular factors, both LPV and Cμ enhancer were transcriptionally inactive. It is thus important to note that these putative positively interacting factors should be supplied from Molt 4 extracts. A negatively acting HeLa cell factor could be the cellular counterpart of the adenovirus type 2 E1A gene products, which have been shown to repress enhancer-induced stimulation of transcription (Borrelli *et al.*, 1984). Although Ephrussi *et al.*, (1985) concluded from their results that enhancer function correlates with

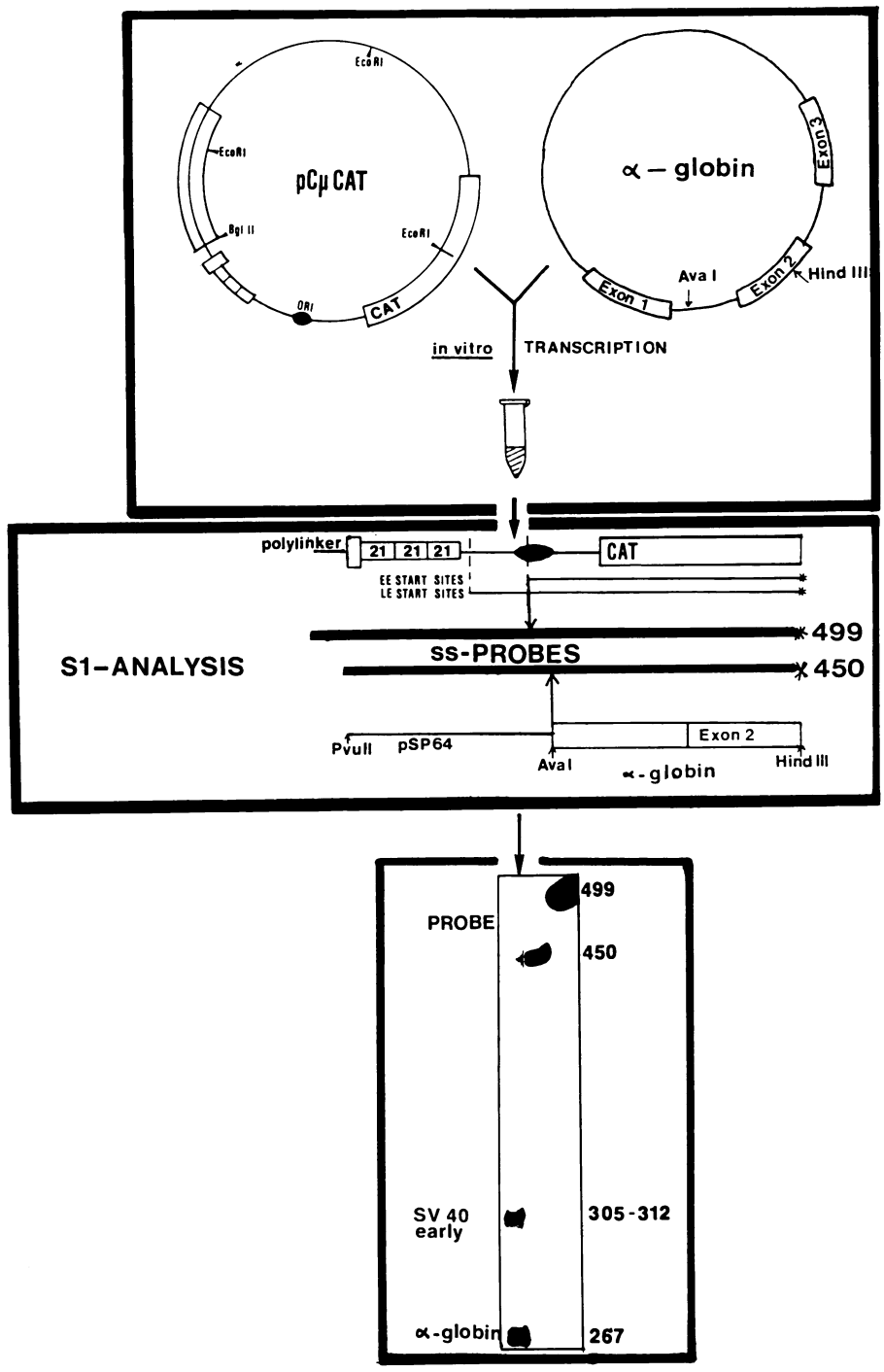

Fig. 7. Analysis scheme for *in vitro* transcription. Test gene (here: pCμ-CAT) and internal standard (α2-globin) were transcribed (top), analysed by the S1 procedure (center) and by polyacrylamide gel electrophoresis (bottom) as described in the text.

positively acting factors it remains to be seen whether their genomic sequencing method was sensitive enough to detect all DNA-binding proteins. Furthermore, no genomic footprint analysis is available using T-cells.

Using appropriate recombinant DNA molecules we are analysing further the exact position of the nucleotide sequence required for this negative effect. However, proteins suppressing transcriptional activity have been described before. Examples include *lac* and *cro* repressors in procaryotes (Miller and Reznikoff, 1978; for review, see Ptashne *et al.*, 1980) and SV40 T-AG (for review, see Tjian, 1981) as well as adenovirus E1A proteins (Borrelli *et al.*, 1984; Velcick and Ziff, 1985, and references therein) in eucaryotic systems. Also, attempts are being made to gather more information concerning the *trans*-interacting factors observed. By the same token, attempts have been started to isolate factors involved in enhancer-mediated transcription. To this end, a nuclear extract from BJA-B cells has been fractionated by column chromatography. One of the fractions shows specific binding activity to the Cμ enhancer (Schlokat *et al.*, personal communication). A combination of purified factors from HeLa, B- and T-cells might lead to the identification and purification of negatively interfering cellular molecules.

Materials and methods

Cell growth

BJA-B (Klein *et al.*, 1975) and MOLT-4 cells (Minowada *et al.*, 1972) were grown in RPMI 1640 medium HeLa cells (Gey *et al.*, 1952) in Spinner medium. Both media were supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were grown in suspension.

Recombinant plasmids

The following plasmids have been described previously: pA10-CAT2 (Laimins *et al.*, 1982), pSV2-CAT (Gorman *et al.*, 1982), pCμ-CAT(5's), pCμ-CAT(5'a), pLPV-CAT(5's), pLPV-CAT(5'a), pLPV-CAT(3's) (Mosthaf *et al.*, 1985), pSV5-CAT (=Mut.5), pSV18-CAT (=Mut.18) (Weiher *et al.*, 1983). pLPV-CAT and pCμ-CAT stand for pLPV-CAT(5's) and pCμ-CAT(5's), respectively. The plasmid containing the 2.2-kb *Pvu*II fragment with the α2-globin gene is a gift from A.Nienhuis.

In vitro transcription and RNA analysis

In vitro transcription reactions were carried out using nuclear extracts of BJA-B, MOLT 4 and HeLa cells. The nuclear extracts were prepared as described previously for HeLa cells (Wildeman *et al.*, 1984). Transcription reactions were performed in a final volume of 20 μl, using a 10-μl extract. Nucleotides were added to give a final concentration of 0.5 mM each; $MgCl_2$ and spermidine trihydrochloride were added as indicated in the figure legends. The DNA optima of the extracts were at 40 μg/ml. KCl was added to 50 mM.

In the competition experiment (Figure 5) 2.5 μg/ml of a plasmid carrying the human α2-globin gene was used as an internal standard. pUC8 was added to achieve the final concentration of 40 μg/ml.

To achieve a considerable ratio of competitor to test gene, fragments of restricted competitor DNA (specific regulatory and unspecific procaryotic DNA) were isolated from native polyacrylamide gels. According to the different lengths of the competitor fragments and to the molar ratios used, unspecific pBR322 DNA fragments (also isolated from polyacrylamide gels) were added to have the same amount of linear DNA in each reaction.

After mixing all of the components at room temperature (addition of the extract last as the final step), the reactions were carried out for 60 min at 33°C. Samples were phenol-$CHCl_3$ extracted, the DNA was then digested with RNase-free DNase I. The transcription products were analysed by S1 nuclease mapping. Briefly, the RNA was resuspended in 10 μl 80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, 1 mM EDTA and mixed with an excess of 5'-^{32}P-end-labeled single-stranded DNA probe. After incubation at 80°C for 10 min, hybridizations were carried out at 42°C for at least 12 h. The mixture was then treated with 70 units of S1 nuclease (Sigma) in 200 μl containing 30 mM sodium acetate pH 4.6, 280 mM NaCl, 4.5 mM $ZnSO_4$ and 30 μg/ml of denatured calf thymus DNA for 90 min at 18°C. S1-resistant DNA was analyzed on 5% polyacrylamide gels containing 5.3 M urea (Maxam and Gilbert, 1980). Using the 499-bp fragment as a probe for the SV40 early promoter, S1-protected fragments of 305 – 312 nucleotides in length according to the early early 5' termini are expected (Tooze, 1981). The 499-bp fragment (*Bgl*II-*Eco*RI of pCμ-CAT) contains a *Bgl*II-*Bam*HI fragment of πVX (Maniatis *et al.*, 1982) that helps strand separation. For analysis of the internal standard, an *Ava*I/*Hind*III fragment of the human α2-globin gene (containing part of the first intron and of the second exon) was cloned into pSP64 (Figure 7). The *Pvu*II-*Hind*III fragment of this construction (450 bp) was used for S1 analysis (Figure 7), the correct fragment being 267 nucleotides in length. The results were quantified by use of an elscript 400 (Hirschman) densitometer. Approximately four out of ten transcriptionally active extracts show an *in vitro* enhancement effect. Among these, a considerable variation from 2- to 15-fold is observed. The reason for this variation remains to be determined.

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