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Negative regulatory sequences in the EIa-inducible enhancer of the adenovirus-2 early EIIa promoter

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ABSTRACT

The adenovirus type 2 early EIIa (EIIaE) transcriptional control region exhibits an EIa-dependent enhancer activity (Imperiale et al., 1985, Proc. Natl. Acad. Sci. USA 82, 381-385). We have determined the sequence requirements for this enhancer activity by analysing the enhancing capacity of the entire EIIa promoter region, or portions of it, when inserted ~ 400 bp upstream of the rabbit β-globin gene. Globin-specific transcription efficiency from the resulting recombinants was measured after transfection into HeLa cells, both in the presence and absence of the EIa products. It was found that the minimal EIIa element with bidirectional, EIa-dependent enhancer activity extends between -111 and -27 relative to the EIIaE major startsite (+1). Furthermore an extensive deletion analysis revealed, within this element, three functionally distinct regions : a central region between about -90 and -70, corresponding to an essential EIIaE upstream promoter element, and two flanking control elements (about 20 bp each) which, in the absence of the EIa products, exert a negative effect on the enhancer activity. Deletion of either one of these control elements renders the EIIaE enhancer activity constitutive, suggesting that the EIa products stimulate the EIIaE enhancer by relieving the negative control mediated by these sequences.

INTRODUCTION

Genetic (1) and biochemical (2) studies have established that the products of the adenovirus immediate early EIa transcription unit are required, during lytic infection, for efficient expression of the other early viral transcription units (EIb, EIIa, EIII and EIV). The induction of these early units has been reproduced in transient expression systems with cloned viral genes (3-7). In the case of the adenovirus type 2 (Ad2) early EIIa (EIIaE) transcription unit, the EIa-induced stimulation has been demonstrated to be mediated at the transcriptional level (8).

The molecular mechanisms by which the EIa products activate transcription are not understood. Structural and functional analysis of the EIIaE promoter region have revealed the existence of two overlapping promo-

ters controlling specific transcription from the major (+1 or EIIaE1) and minor (-26 or EIIaE2) startsites, respectively (9). While specific sequences have been implicated in the EIa-mediated stimulation of initiation from the minor EIIaE2 promoter (9), no unique sequence element responsible for the EIa-response of the major EIIaE1 promoter has been as yet identified (9-11). Recent reports (12, 13) have shown that a DNA fragment spanning the EIIaE promoter region from position -262 to -21 was able, in the presence of the EIa gene products, to stimulate the transcriptional activity of homologous or heterologous promoter elements, indicating the existence of an EIa-dependent enhancer element.

In parallel studies, we have analyzed the effect of a set of EIIaE promoter fragments on globin-specific transcription, when inserted in either orientation in front of an entire rabbit β-globin gene, extending from position -425 to +1700 (with respect to the globin gene capsite). The various recombinants were assayed for transcription from the globin gene promoter in a transient expression system in the presence and the absence of the EIa gene products. The globin gene, used here as an heterologous test-gene for EIIaE enhancer activity, was chosen for the following reasons : (i) its promoter structure has been well characterized and maximal promoter activity shown to be contained within 109 bp of 5'-flanking sequences (14), (ii) it is transcribed at a low, but detectable, efficiency after transfection into HeLa cells and its transcription rate is only moderately increased (relative to that of the EIIaE transcription unit) after cotransfection with an EIa-containing recombinant (see Results). The results indicate that the minimal EIIaE promoter fragment providing maximal, bidirectional and EIa-inducible enhancer function extends from position -111 to -27. In addition the present study reveals the existence, within this DNA segment, of negative regulatory sequences located on each side of an element (situated approximately between -90 and -70) essential for EIIaE "constitutive" promoter function. These observations raise the interesting possibility that the EIa gene products stimulate EIIaE promoter activity by relieving the repression mediated by these negative regulatory sequences.

MATERIALS AND METHODS

Cells and transfection : HeLa cells grown in monolayers in 10 cm petri dishes were transfected at 50% confluence by the calcium phosphate coprecipitation technique as described (15). The various recombinant DNAs were transfected as indicated in the figure legends and the final amount of DNA

was adjusted to 15 µg per plate with M13mp8 RF DNA.
S1 nuclease analysis : After 20 h of contact with the DNA coprecipitate the cells were washed, and fresh medium was added. Cells were collected 12 h later and total cytoplasmic RNA was prepared after lysis with 0.5 % Nonidet P40 as described (16). The purified RNA was hybridized with an excess of 5'end ^{32}P-labeled single stranded DNA probes and subjected to S1 nuclease digestion (for 2 h at 25°C) as described (17). Resistant DNA fragments were analyzed on 8% acrylamide urea sequencing gels (18).

Quantitation of the amounts of specific transcripts was achieved by densitometric scanning of the autoradiograms after several exposures of each gel and the results, computed from 2 to 5 separate transfection experiments, using different plasmid preparations, are presented below typical autoradiograms (Figures 2-5). In all experiments the level of EIa transcription, from either the pEIA$^-$ or pEIA13S recombinants which were cotransfected with the pG derivatives, was determined to ascertain that similar transfection efficiencies were observed throughout each experiment. Standard deviations calculated for each E and SE value (see Figures) were in the range of 10 to 20%.

RESULTS

The enhancer assay.

To test for enhancer properties of sequences present in the EIIaE promoter region we constructed the pG test-plasmid (Fig. 1) where defined restriction fragments can be easily inserted in a polylinker sequence placed 425 bp upstream of the capsite of the entire rabbit β-globin gene. The resulting recombinants (pG derivatives) were introduced into HeLa cells by the calcium-phosphate coprecipitation technique and the globin-specific transcription (GLOB bands in figures) was analysed by quantitative S1 nuclease mapping (see Materials and Methods).

The enhancer potential of the various sequences introduced in the polylinker was analyzed by their ability to activate transcription from the heterologous globin promoter. Since EIIaE promoter activity is itself regulated by the EIa early gene products, the enhancer activity of the EIIaE sequences was assayed both in the presence and in the absence of these products. Thus, the pG derivatives were cotransfected with either an EIa-cDNA recombinant producing the EIa 13S mRNA (pEIA13S, see Fig. 1) or an EIa-defective recombinant containing the whole EIa 5' region, in order to keep the EIa promoter concentration constant under both conditions (pEIA$^-$, see legend to Fig. 2). The pEIA13S recombinant was chosen, rather than a plasmid bearing the entire EIa transcription unit (and producing both 13S and 12S EIa mRNAs), because it gave the most reproducible, and generally the highest, level of EIIaE-specific transcriptional stimulation (8). To limit as much as possible any competition between the weak globin and strong EIa promoters, a molar ratio of one to ten of the pEIA to the pG

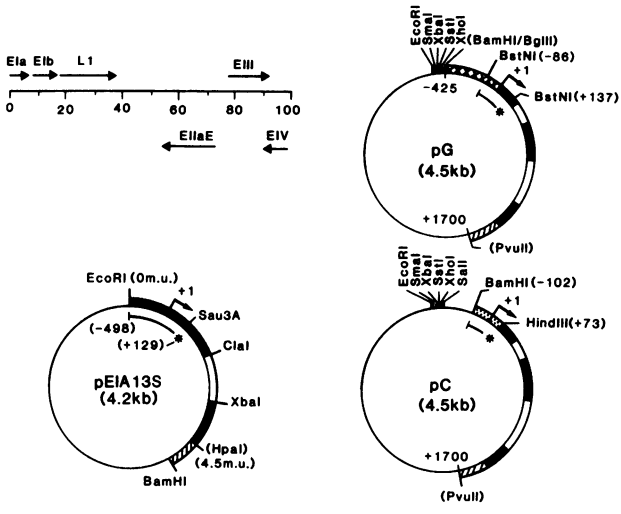

Figure 1 : Organization of early adenovirus transcription units and structure of the pEIA13S, pG and pC recombinants.
A genomic map of adenovirus DNA showing early viral transcription units (except EIIb) is given (taken from data in 19). The pEIA13S recombinant derives from the pEIASV plasmid (described in 3). It contains the leftmost HpaI fragment of the Ad2 genome (black boxes) in which the ClaI-XbaI segment (open box) has been replaced by the corresponding intronless segment of a cDNA clone complementary to the 13S transcript of EIA (20). The hatched box correspond to the 135 bp SV40 HpaI-BamHI fragment containing the SV40 early and late polyadenylation signals. The DNA probe used for S1 nuclease mapping of the transcripts produced from this plasmid is the coding strand of the Sau3A-EcoRI fragment (+129 to -498 with respect to the Ela capsite), labeled with ^{32}P at the 5'end.

The pG recombinant has been constructed by inserting a BglII-PvuII fragment of the rabbit β-globin gene (from positions -425 to +1700, with respect to the globin capsite) between the BamHI (in the polylinker) and PvuII site of a pBR322 derivative containing the M13mp12 polylinker between the EcoRI and HindIII sites. The black and open boxes depict the exon and intron sequences, and the crossed and hatched boxes correspond to the upstream and downstream non-transcribed sequences, respectively. The DNA probe used for S1 nuclease mapping of the globin transcripts is the 5'end-labeled coding strand of a BstNI-BstNI fragment (-86 to +137 with respect to the globin capsite).

The pC recombinant derives from pBW7.8 and pBW5 (21). It contains the conalbumin promoter from position -102 to position +62 (stippled box), with respect to the conalbumin capsite, flanked by BamHI and HindIII linkers (10 bp-long) and placed in front of the rabbit β-globin gene extending from position -9 to position +1700, with respect to the globin capsite (symbols are as for pG). The vector sequences are the same as for pG, except that a 275 bp long SalI-BamHI pBR322 fragment, with a XhoI linker next to the SalI site, was inserted between the XhoI and the BamHI sites of the M13mp12 polylinker. The DNA probe used for S1 nuclease mapping of the transcripts initiating at the conalbumin startsite is the 5'end-

labeled coding strand of the BamHI-HindIII fragment (-102 to +73). The arrows indicate the origin and direction of transcription, the DNA probes are schematically represented inside the plasmids and the restriction sites lost during cloning are in parentheses.

recombinants was used in all transfection experiments (see Materials and Methods). Under these experimental conditions the globin-specific expression was generally stimulated about 3-fold (between 1.5 and 5-fold, depending on the plasmid preparation) by the EIa 13S mRNA product, while transcriptional stimulation from the EIIaE promoter was about 15-fold (unpublished data). Only those experiments showing equal EIa transcription (see EIA signal) were taken into account within series cotransfected with either pEIA$^-$ or pEIA13S. As previously reported (8), the 10-fold lower amount of cytoplasmic RNA originating from pEIA$^-$, compared to pEIA13S, is related to a lower stability of the shorter pEIA$^-$ specific transcripts. The intensity of these signals was therefore estimated on autoradiograms from longer exposures than those shown in Fig. 2-5.

Since the efficiency of the globin promoter in the pG plasmid is by itself sensitive to the EIa gene products, the globin-specific transcription levels obtained with the various recombinants were expressed in each case relative to the value found for pG under the same cotransfection conditions. This gives the enhancement factor (E) provided by the inserted EIIaE fragments. The factor (S.E.) by which the EIa product stimulates the enhancer function corresponds to the ratio of E values obtained in the presence and absence of the EIa product. The E and S.E. values deduced from the quantification of separate experiments (see Material and Methods) are given in Fig. 2-5 below the most representative autoradiograms.

The EIIaE promoter behaves as an enhancer element in the presence of the EIa gene products.

A DNA fragment, extending from position -249 to +37 with respect to the EIIaE major startsite and spanning the entire EIIaE promoter region (3, 9) was inserted in both orientations in the pG plasmid, generating pGE and pGEi recombinants (Figure 2). In the following discussion, the orientation where the EIIaE +1 site is the closest to the globin +1 site will be called the "direct" orientation and the other one, the "reverse" orientation.

Insertion of this fragment had very little, if any, effect on globin-specific transcription when the resulting recombinants were transfected in the presence of pEIA$^-$ (compare E values for lanes 1 to 3). In contrast,

cotransfection with pEIA13S resulted in a 12 and 13.5-fold stimulation of transcription, in the case of pGEi and pGE, respectively (compare lanes 2 and 3 with lanes 5 and 6),while there was only a 3-fold stimulation in the case of the parental pG plasmid (compare lanes 1 and 4). Corrected for the basal level of globin transcriptional stimulation by the EIa gene product (as measured on the pG plasmid), these results indicate that, irrespective of their orientation relative to the globin gene, the EIIaE sequences stimulate globin transcription about 4-fold in the presence, but not in the absence (pEIA$^-$) of the EIa 13S mRNA product (see E and S.E. values in Fig. 2). The EIIaE sequences exert their effect in cis, since there was no additional stimulation of globin transcription, above that achieved by the EIa gene products on pG, when the EIIa sequences were carried by a separate plasmid (result not shown).

Negative control sequences flank the minimal EIIaE enhancer element.

To delimit more precisely the EIIaE promoter element involved in the EIa-dependent enhancer function, sequences were progressively deleted from the 3' and 5' borders of the EIIaE promoter and the truncated fragments inserted in both orientations into the pG test-plasmid.

When the EIIaE sequences were placed in the "direct" orientation and trimmed from their 3'-end, maximal EIa-induced enhancer activity was preserved up to position -27 with respect to the EIIaE major startsite (pGL-27), while deletions extending further 5' reduced it gradually (compare E values for lanes 8 to 12 in Fig. 3A). These results place the 3' border of the EIa-dependent enhancer element, in this orientation, around position -27. It is noteworthy that the E values found for the pGL series were reproducibly higher than that obtained with the pGE recombinant (compare Fig. 3A, lanes 8 to 12 and Fig. 2, lane 6). This difference can be attributed to the fact that the EIIa element present in pGE comprises the entire promoter region, including the EIIaE start site. A significant part of the transcriptional enhancement is therefore diverted to this most proximal start, thereby reducing the effect on the more distal globin start. Consequently, deletion of the EIIa startsite region, as in the pGL series, results in a stronger enhancer effect on globin-specific transcription.

When the EIIaE sequences were progressively deleted from the 5' end, the EIIa enhancer activity became independent of the EIa product after deletion to position -87 (compare E values in Fig. 3B, lanes 6 and 13). Deletions extending further 3' gradually impaired the enhancer activity,

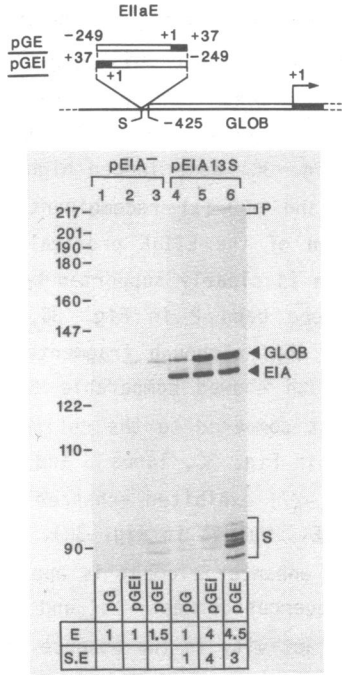

Figure 2 : Enhancer effect of the entire EIIaE promoter region placed at distance upstream of the rabbit β-globin gene :
The diagram on the top depicts the pGE and pGEi recombinants which were obtained by insertion in either orientation, into the SmaI site (S) of pG, of a SmaI-HincII fragment taken from the Mp9EII3' recombinant (9) where the EIIaE sequences extend from positions -249 to +37, with respect to the major EIIaE1 startsite. The black portions of the diagram correspond to the first leader or exon of the EIIaE or globin genes respectively, with the transcription startsites marked (+1). The arrow indicates the direction of globin transcription. Five μg of pG (lanes 1, 4), pGEi (lanes 2, 5) or pGE (lanes 3, 6) were cotransfected into HeLa cells with 0.5 μg of either pEIA⁻ (lanes 1-3) or pEIA13S (lanes 4-6). The pEIA⁻ recombinant is identical to pEIA13S (see Fig. 1) but lacks the EIa coding region between positions +129 and + 1075 (22). Specific EIa and globin transcripts were analysed by the S1 nuclease assay (see Methods), using the corresponding probes described in Figure 1 and 6 μg-samples of RNA. The autoradiogram of a typical gel is shown : GLOB and EIA refer to probe fragments protected by specific transcripts initiated at the β-globin and EIa capsites (+1) respectively (depending on the specific activity of the EIa-probe, or on the exposure of the autoradiograms, additional EIa-specific bands appeared between the major EIA and GLOB signals; see Fig. 3-5); P refers to the full-length globin probe protected by transcripts initiated upstream from the BstNI site at position -86 (see pG in Figure 1); S refers to a set of globin probe fragments protected by transcripts initiated upstream from this site (see text) and spliced to pseudo-acceptor sites located between positions +42 and +48 (14). Numbers on the left correspond to the length in nucleotides of ^{32}P-end-labeled MspI restriction fragments of pBR322 used as DNA size markers. The E and SE values at the bottom refer to the globin-specific transcripts as defined in the text.

which was abolished upon removal of sequences up to -62. To eliminate possible interpretation problems linked to this competition between the adenovirus and globin initiation sites, we have examined the enhancer function of EIIaE fragments lacking the EIIaE startsite region. The E values found for pGP-86 and pGP-111 (Fig. 3C) were indeed higher than those found for the corresponding pGD-87 and pGD-111 recombinants, again probably as a consequence of the deletion of the EIIaE proximal promoter region in the pGP series (this conclusion is clearly supported by the lower intensity of the full-length globin probe band P in Fig. 3C, compared to Fig. 3B). Furthermore, it is evident that although fragments extending from -111 to -27 or from -86 to -27 both showed comparable enhancer activity in the presence of the EIa product compared to the entire promoter fragment from -249 to -27 (see E values in Fig. 3C, lanes 5 and 6 and Fig. 3A, lane 9), only the shortest (-86 to -27) exhibited enhancer activity in the absence of the EIa product (see S.E. values in Fig. 3C). Therefore, the 5' limit of the EIa-dependent EIIaE enhancer element is apparently located close to position -111, with the sequences between -111 and -86 exerting an inhibitory effect on the enhancer activity in the absence of the EIa products.

When placed in the "reverse" orientation, analysis of EIIaE 3'-deletions (pGLi series) also revealed a negative control element, this time located closer to the EIIaE major startsite. Compared to the pGEi recombinant bearing the entire EIIaE promoter (Fig. 2, lane 2), deletions up to position -48 slightly increased (2-fold) globin template activity (Fig. 4A, lanes 5-7), probably due to a competition between the EIIaE and globin promoters (see above). On the other hand, deletion of the EIIaE sequences to position -61 and -71 produced a marked increase in the globin signal (4.5 and 7-fold respectively, lanes 3 and 4, Fig. 4A), indicating that, in this orientation, the sequences between positions -48 and -71 exert a negative effect on the flanking EIIaE enhancer element.

To define the 5' border of the minimally active EIIaE fragment, sequences were removed from positions -249 to -86, keeping the 3' border at position -71 (Fig. 4B). It appears (lane 5 to 8) that, in this orientation, most of the enhancer capacity is retained by an EIIaE fragment extending from positions -71 to -111, setting, in this orientation, the 5' limit of the EIIaE enhancer element close to or slightly 5' of position -111.

Altogether, these results indicate that the two EIIaE sequences, which directly surround the central element (between -87 and -71) of the

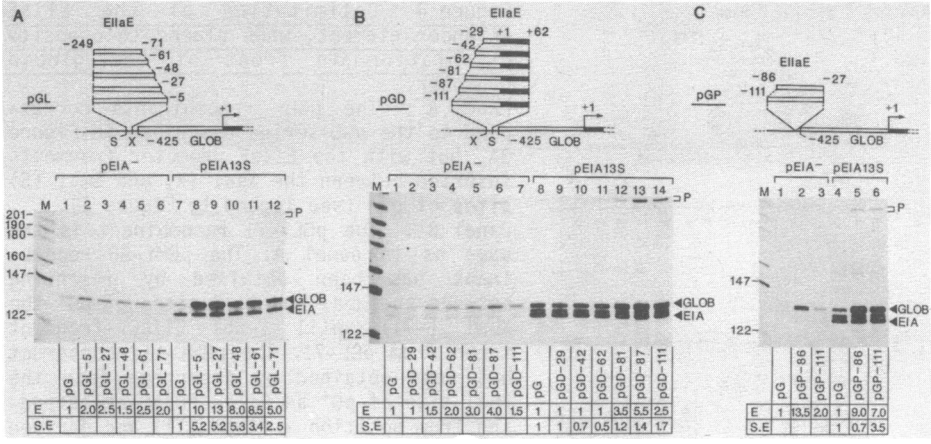

Figure 3 : Delimitation of the EIIaE enhancer element, when placed 5' to the globin promoter and in the same orientation.
Panel A : The pGL series has been obtained by inserting 3'-deleted EIIaE promoter fragments between the SmaI (S) and XbaI (X) sites of pG (see Figure 1). These fragments are limited on their 5' end by the SmaI site at position -249 and on their 3' end by an XbaI linker which was ligated to the 3' deletion end-points of the Mp9EII 3' recombinants (9).
Panel B : The pGD recombinants have been constructed by inserting 5'-deleted EIIaE promoter fragments between the XbaI (X) and SmaI (S) sites of pG' (a plasmid identical to pG, but with the SmaI and XbaI sites reversed). In pGD-87, -81, -62, -42 and -29 these fragments are limited on their 3' end by the PvuII site at position +62 and on their 5' end by an XbaI linker which was ligated to the 5' deletion end-points of the Mp8EII5' recombinants (9). In pGD-111 the EIIaE fragment was derived from the pBX-111 deletion mutant (3) after addition of an XbaI linker, 5' to the BamHI site present at the deletion end-point.
Panel C : The pGP-86 recombinant has been obtained by inserting, between the SmaI and XbaI sites of pG, the HaeIII (-86)- XbaI (-27) EIIaE fragment taken from pGL-27. The pGP-111 recombinant has been obtaind by inserting into the XbaI site of pG an EIIaE fragment extending from positions -111 to -27 and derived from pGL-27 and pGD-111 by recombination at the unique NarI site (at position -95 in the EIIaE fragment).
Symbols, transfection conditions of pG or each pG derivative and representation of the results are as in Figure 2.

enhancer, negatively control its function. Each flanking element inhibits the enhancer activity only in the orientation where it is situated the furthest away from the test-gene (Fig. 6B and D).

The EIa gene products prevent the inhibition of the EIIaE enhancer element by the negative regulatory flanking sequences.

As shown in Figure 3 by the corresponding E values, the enhancer function of EIIaE fragments retaining the -111 to -86 inhibitory element

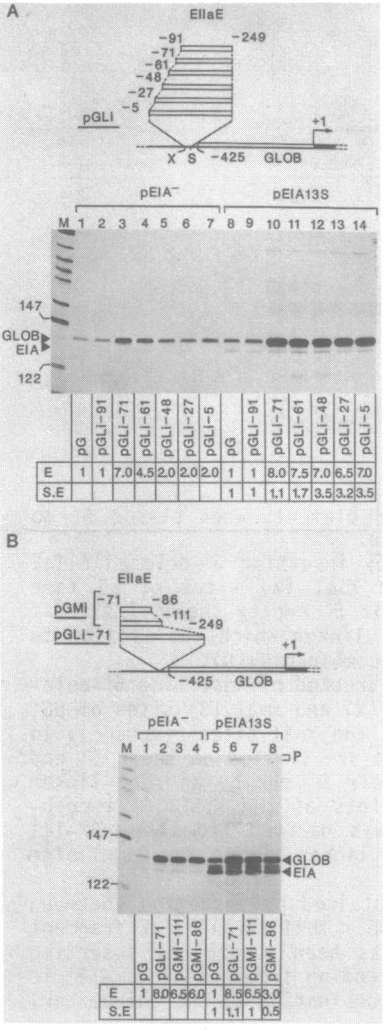

Figure 4 : Delimitation of the EIIaE enhancer element, when placed in opposite orientation in front of the globin promoter.
Panel A : The pGLi recombinants correspond to the pGL series described in Figure 3A, but with the EIIaE promoter fragments inserted between the XbaI (X) and SmaI (S) sites of pG' (see legend to Figure 3).
Panel B : The pGLi-71 recombinant is the same as in panel A. The pGMi-86 recombinant has been obtained by inserting between the XbaI and SmaI sites of pG' the XbaI (-71)-HaeIII (-86) EIIaE fragment taken from pGL-71. The pGMi-111 construct has been obtained by inserting into the XbaI site of pG' an EIIaE fragment extending from position - 71 to -111 and derived from pGLi-71 and pGD-111 by recombination at the unique NarI site (position -95 in the EIIaE promoter).
Symbols, transfection conditions of pG or each pG derivative and representation of the results are as in Figure 2. Note that the EIa-specific transcripts were analysed with probes of different specific activities in panels A and B.

(pGL series and pGP-111) was activated by the EIa products to about the same level as that exhibited by an EIIaE fragment lacking that element (pGP-86), in the absence of the EIa product. Furthermore, the enhancer activity of this latter EIIaE fragment was not significantly affected by EIa cotransfection, as indicated by the S.E values which were close to 1. Similarly, comparison of the S.E factors corresponding to the various pGLi recombinants (Fig. 4A), in which the EIIaE sequences are in the "reverse"

orientation, revealed that deletion of the regulatory sequences from positions -48 to -71 rendered the enhancer element independent of the EIa gene product. As expected, the enhancer function of the minimal EIIaE active fragment present in pGMi-111 did not require the EIa product to stimulate globin expression (see corresponding S.E factor in Fig. 4B).

It appears therefore that the EIa gene product stimulates the EIIaE enhancer activity by relieving the negative effect of the flanking regulatory elements. The EIIaE promoter region between positions -111 to -27 constitutes the minimal element with EIa-dependent bidirectional enhancer activity, as confirmed by the analysis of the pGP-111 and pGPi-111 recombinants (Fig. 5A, lanes 1-6) in which this minimal enhancer element is clearly induced by the EIa gene product, and about equally active in both orientations.

Comparison of the EIIaE and the simian virus 40 enhancer elements.

To evaluate the relative enhancer efficiencies of the minimal EIIaE element defined above and the well documented simian virus 40 (SV40) enhancer, we have inserted this latter element into the pG plasmid in place of the EIIaE element (pGB, Fig. 5A). Comparison of the globin signals in lanes 5 to 7, after correction for the 10-fold lower amount of RNA analysed in lane 7, revealed that the SV40 enhancer element was at least 50-fold more active than the EIa-induced EIIaE element (compare E values in lanes 5-7, Fig. 5A). In these experiments transcription of the pGB plasmid was analysed in the absence of the EIa products. As expected from earlier studies (19, 20), when the SV40-enhancer-containing plasmid was cotransfected with pEIA13S, a 10-fold decrease in the SV40 enhancer efficiency was observed (compare lanes 7 and 8, Fig. 5A). Therefore, the two enhancer elements not only differ in their relative strength but also in their response to the EIa gene products.

The EIIaE enhancer effect is not restricted to the globin promoter.

To exclude the possibility that the effect of the EIIaE enhancer was restricted to the globin promoter, this element was inserted in both orientations, about 300 bp upstream from a fragment of the chicken conalbumin gene promoter, fused to the globin coding sequences (see Fig. 1 and 5B). The resulting pCE and pCEi recombinants were transfected in the absence or presence of the EIa 13S mRNA product, and the conalbumin-specific transcripts (CON) were analysed (Fig. 5B). As indicated by the E values (lanes

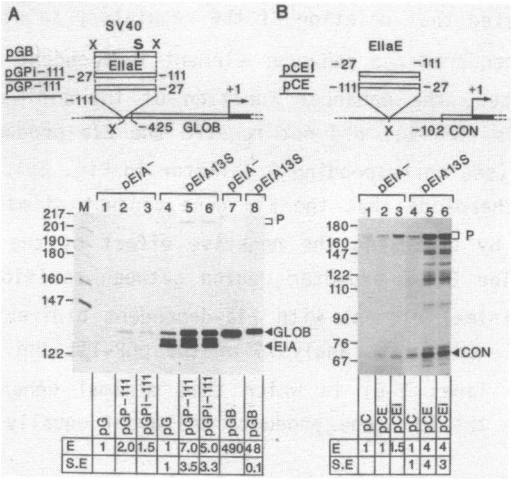

Figure 5 : Comparison of the effect of the EIIaE and SV40 72 bp enhancer elements on the globin promoter efficiency and effect of the EIIaE enhancer element on the conalbumin promoter function.
Panel A : The pGP-111 recombinant is the same as in Figure 3C. The pGPi-111 recombinant is the same as pGP-111, but with the EIIaE fragment inserted in the reverse orientation. The pGB recombinant has been constructed by inserting, into the XhoI site (X) of pG, an SV40 DNA fragment flanked by XhoI linkers (X) and containing a single 72 bp enhancer element. This fragment, which derives from a mutant SV40 DNA with a BamHI site at coordinate 101 and with a deletion between the two SphI sites (coordinates 128 and 200) (23), extends from this BamHI site to the next PvuII site (coordinate 270). The fragment has been inserted in the direct orientation with respect to the globin gene as indicated by the position of the SphI site (S) at coordinate (200).
Symbols, transfection conditions and representation of the results are as in Figure 2, except that only 0.6 g of total cytoplasmic RNA was used for the S1 nuclease assay in lanes 7 and 8 (the E values were corrected accordingly).
Panel B : The pCE and pCEi recombinants were obtained by inserting in either orientation into the XbaI site (X) of pC (see Figure 1) the EIIaE fragment of pGP-111. Five g of pC (lanes 1, 4), pCE (lanes 2, 5) or pCEi (lanes 3, 6) were cotransfected with 1 g of either pEIA⁻ (lanes 1-3) or pEIA13S (lanes 4-6). Specific conalbumin transcripts were analysed by the S1 nuclease assay as in panel A but using the conalbumin probe shown in Figure 1 and 20 g of total cytoplasmic RNA. CON refers to probe fragments protected by specific transcripts initiated at the conalbumin capsite (+1) and P refers to the full-length conalbumin probe protected by transcripts initiated upstream from the BamHI site at position -102 (see pC in Figure 1). The E and SE values refer to the conalbumin-specific transcripts as described in the text.

1-6) and the corresponding S.E factors, the response of the conalbumin transcription signal to the EIIa element and the EIa product is very similar to that of the globin signal (compare pCE and pGP derivatives in Fig. 5A and B).

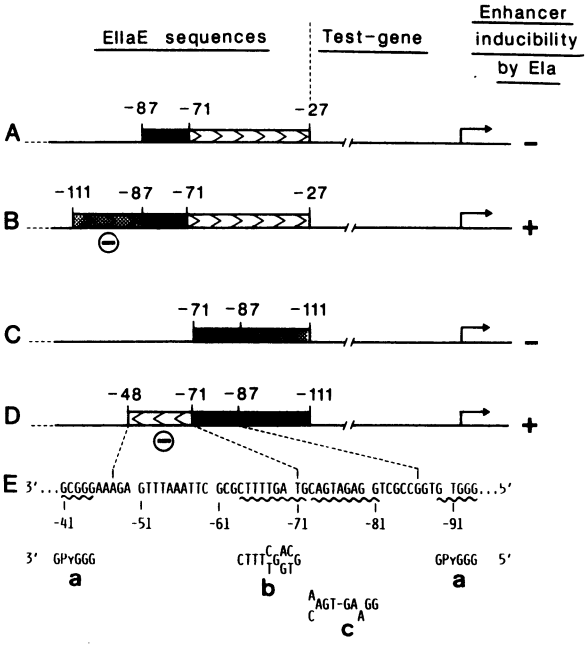

Figure 6 : Summary of the EIIaE enhancer properties
The EIIaE sequence elements involved in the enhancer activity and defined from figures 3 and 4 are represented in either the "direct" (A and B) or the "reverse" orientation (C and D) with respect to the test-gene. The origin and direction of the corresponding test-transcription is shown by the arrow, and the EIa-dependence of the enhancer activity indicated by the + (EIa-inducible enhancer) or - (EIa-independent or -constitutive enhancer) signs. The (-) symbol refers to the negative regulatory EIIaE element involved in the "repression" of the enhancer activity in each orientation (B and D). The EIIaE nucleotide sequence between positions -41 and -94 is given (E) and relevant homologies with other adenovirus promoter sequences (see text) are underlined. The corresponding consensus sequences are outlined below. Sequence a has been found in related positions in the EIb, EIII and EIV promoters (49), sequence b, is also present in the EIIIand EIV promoters (7), and sequence c has been identified in EIa and EIV enhancer-like elements (38, 39).

DISCUSSION

What distinguishes an enhancer from an "ordinary" upstream promoter element?

Comparison of the structural organization of a number of eukaryotic class B promoters has led to a general view of upstream promoter and enhancer elements as being distinguished both by their position and their function within the promoter (26). Enhancer elements are usually located at more than 100 bp, either upstream or downstream from the capsite of the

activated promoter, while upstream elements are positioned next to the consensus or substitute TATA box element, generally between positions -110 and -40. The major functional criteria fulfilled by all enhancers is their ability to activate specific transcription over long distances from the promoter, even though maximal activation is observed when they are closest to the capsite (21). On the contrary, the distance between the upstream elements and the TATA box appears to be much less flexible, since insertion of spacer DNA sequences larger than 50 bp, between these two elements of the HSV thymidine kinase, the rabbit β-globin, and the SV40 early promoters, drastically reduced transcription efficiency (27-29). The other properties shared by enhancers, namely their bidirectionality and ability to activate non-homologous promoters, are less specific. Upstream elements of the SV40 and thymidine kinase promoters have indeed been shown to stimulate transcription in both their natural and inverse orientations (30, 31). On the other hand elements present in the adenovirus EIa far-upstream promoter region fulfill all the criteria of enhancers, but function only in their natural orientation (32). Finally, examples of upstream elements which are interchangeable between different promoters are found in the case of the thymidine kinase and β-globin genes (29), the SV40 21 bp repeat and the adenovirus major late promoter (33) and the adenovirus EIIa early, EIIa late, EIII and major late promoters (34).

The data presented here show that the EIIaE element located at a position typical for "ordinary" upstream promoter elements, but which can be moved at least 4000 bp away from the EIIaE capsite (12), meets the definition of a true enhancer element. The strength of this element, which is about 50-fold less efficient than the SV40 72 bp repeat, is low, but comparable to that of other enhancers like those found in the MLV long terminal repeat or in the adenovirus EIa unit which are about 25 and 10-fold weaker than the SV40 enhancer, respectively (32, 35). In this respect, it is interesting that the SV40 21 bp repeat does not stimulate globin transcription when placed at the same position as the EIIaE element, in the same vector (our unpublished result).

Besides the EIIaE promoter, the mouse metallothionein gene offers another example where an upstream promoter element (between -63 and -188) exhibits typical enhancer properties (36). The fact that the enhancer activity of this element is stimulated by heavy metal ions (36) and that the metal responsive elements flank an essential upstream sequence (between -70

and -90) (37), suggests striking similarities between these two inducible promoters.

Sequences required for efficient constitutive EIIaE transcription contribute to the EIIaE enhancer activity.

Earlier studies (3, 11, 12) have shown that about 90 bp of EIIaE 5'-flanking promoter sequences were sufficient for maximal EIIaE-specific constitutive transcription. Transcriptional analysis of internal deletion (9) and linker-scanning mutations (9, 11) have revealed distinct promoter elements within this region, including one located between positions -90 and -70, which is required for efficient initiation of transcription from both the major and minor EIIaE startsites. It is clear that this sequence plays an essential role in the EIIaE enhancer function described here, since a fragment extending from -86 to -71 exhibits enhancer activity when placed in the "reverse" orientation in front of the globin gene. Interestingly this element shares some sequence homology (see Fig. 6E) with enhancer-like elements found in the adenovirus EIa (38) and EIV (39) promoter regions (5'-GGAAGTGAC-3'). In the "direct" orientation the gradual decrease in enhancer activity observed by the successive removal of the sequences between -27 and -48 and between -61 and -71 (Fig. 3A, lanes 8-12) corresponds to the deletion of two different EIIaE promoter elements (9). Characterization of the exact relationship between these EIIaE promoter elements and the enhancer function requires further investigation.

Induction of the EIIaE enhancer activity by the EIa products corresponds to the relief of a negative control.

In this report we show that elements between -111 and -87 and between -71 and -48 are involved in the inhibition of the EIIaE enhancer effect in the absence of the EIa products (Fig. 6B and D). Deletion of either of these elements activates the enhancer activity in one direction. Bidirectional activation by the intact enhancer element is obtained in the presence of the EIa products, indicating that these products simultaneously relieve the "repression" exerted by both control regions. The inhibitory effect could be due to the binding of one or several specific repressor molecules to both regulatory sites (-111/-90 and -70/-48), thereby sterically impairing the access of transcription factors to the essential upstream promoter element (-90/-70). The participation of short-lived repressor molecules in the control of adenovirus early gene expression has been previously suggested by experiments in which cells were treated with pro-

tein synthesis inhibitors prior to or shortly after infection. The treatment resulted in an activated early gene transcription (40-42). However, the involvement of such repressor molecules has been questioned (43). The possibility exists that the regulatory sequences directly inhibit the EIIaE transcription, by lowering the affinity of the transcription factors for the upstream region.

The Eia gene products could therefore induce the enhancer activity either by inactivating the repressor molecules or by catalyzing the efficient binding of specific transcription factors to the EIIaE upstream promoter region. This could be achieved by direct interaction of the Eia products with the transcription machinery or by raising the concentration of active transcription factors (44). In the absence of the Eia products, deletion of either of the control sequences could destabilize the interaction of the repressor molecule(s) with the remaining control element, and therefore stimulate the enhancer activity. That this stimulation occurs essentially over the non-deleted region of the truncated enhancer is most likely related to the fact that both control elements contribute themselves, each in an opposite direction, to the enhancer activity (see Results). Therefore, deletion of either of these elements renders the enhancer activity constitutive (i.e. independent of the Eia products) in one direction, but impairs its function in the other one.

Sequence components mediating transcriptional repression have recently been observed in the upstream region of yeast gene promoters (for refs., see 45, 46), in the hormonally-regulated ovalbumin gene (47) and in the vicinity of the mouse heavy chain immunoglobulin enhancer (48). Together with the enhancers, these regulatory elements may contribute to the numerous combinatorial possibilities which are required for gene regulation in complex organisms.

The requirement of defined sequence elements for stimulation of the EIIaE enhancer by the Eia products was unexpected. Indeed, studies using linker-scanning mutations of the EIIaE promoter did not reveal any unique discrete sequence element implicated in the Eia-mediated control of transcription from the EIIaE major startsite (9, 11). However 5'-external deletions of EIIaE promoter sequences to about position -60 have been shown to drastically reduce the basal level of EIIaE expression and virtually to abolish its inducibility by the Eia gene products (3, 11, 13), suggesting that essential promoter elements required for both constitutive and Eia-

regulated transcription are located upstream from this position. It is noteworthy that the control region between -71 and -48 contains an element required for the EIa-mediated stimulation of transcription from the EIIaE minor startsite (9). The observation that an external deletion to position -86 reduced only by about 30% the extent of the EIa-mediated induction (11), and did not increase basal EIIaE transcription, raises the possibility that in its natural location (in the EIIaE upstream region), the proximal regulatory element (-71/-48) is by itself sufficient to repress the transcription in the absence of the EIa products. It is likely therefore that the enhancer control elements (-111/-86 and -71/-48), which we described here, are involved in the mechanism of EIIaE transcriptional induction by the EIa products. The presence, within these control regions (see Fig. 6E), of sequence components homologous to elements found at similar positions in the adenovirus EIb, EIII and EIV promoters (7, 49) suggests that the control mechanisms of these genes by the EIa products could be similar. It is tempting to speculate that the peculiar EIIaE promoter structure, where the various control elements are intermingled within a 100 bp DNA fragment, is related to its location in the viral genome, between the divergently transcribed EIIa and EIII transcription units, separated by only about 500 bp. It will be interesting to examine the function of this EIIaE enhancer activity during the progression of the lytic infectious cycle on both of these EIa-regulated transcription units.

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