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Specific cellular proteins bind to critical promoter sequences of the adenovirus early EIIa promoter

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ABSTRACT

As an approach to the identification of essential factors required for specific expression of the adenovirus type 2 EIIaE early (EIIaE) promoter, an in vitro system was established. Under appropriate conditions, using crude extracts of non-infected HeLa cells, efficient and accurate EIIaE expression has been reproduced. As in vivo, this transcription was strongly dependent upon the integrity of two non-consensus TATA-like elements, T1 and T2, corresponding to the major (EIIaE1) and minor (EIIaE2) startsites, respectively, as well as upon intact upstream elements (A, between -40 and -50 and B, between -70 and -90) common to both overlapping promoters. The implication of specific DNA-binding proteins in the transcriptional effects mediated by these elements was demonstrated by DNAse I footprinting analyses. Both crude nuclear extracts and partially purified fractions confer specific protection against DNAse I digestion to the T1 and B promoter elements defined above, and to a far upstream region (element C, between -110 and -150), which has previously been identified as a weaker promoter element by in vivo transcriptional studies. Separation of the T1 recognition factor from those which bind to the upstream elements B and C by chromatographic fractionation of the extracts has also been achieved.

INTRODUCTION

It is well established that eukaryotic gene expression is largely regulated at the level of transcriptional initiation. Cis-acting DNA sequences, which are critical for selective and efficient transcription have been defined (for reviews, see 1-6). The involvement of specific protein factors in transcriptional control has also been documented using footprint analyses (7) and nucleoprotein electrophoretic retardation studies (8).

An interesting system for the study of gene control is that of the adenovirus early EIIa (EIIaE) transcription unit (TU). This TU is controlled by two overlapping promoters, which direct initiation of transcription from two startsites : the major, EIIaE1, at position +1, and the minor, EIIaE2, at position -26, neither one of which is preceded by a consensus TATA-box sequence (9). Furthermore, like that of the other adenovirus early TU (EIb, EIII and EIV), transcription of the EIIaE TU is regulated by the immediate

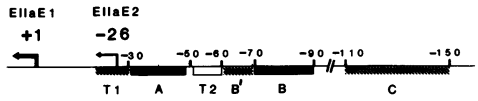

Fig. 1 : Essential EIIaE sequence elements required for constitutive in vivo promoter activity.
The EIIaE promoter region is depicted, with the arrows pointing to the direction of transcription (according to the conventional adenovirus transcription map) and marking the position of major, EIIaE1 (+1), and minor, EIIaE2 (-26), startsites. The promoter elements unique to EIIaE1 promoter function are represented by the hatched boxes, that unique to the EIIaE2 promoter function by the open box, and those involved in both activities by the closed boxes. The delimitations of these elements is deduced from earlier work (17, 15).

early EIa gene products (10-12). Hence, the EIIaE TU can be used as a model for the identification of the sequences and protein factors which interact within an atypical promoter, and also for the study of the mechanism of EIa-mediated transcriptional induction.

The promoter elements required for constitutive EIIaE transcriptional activity have been identified by analysing the in vivo template efficiencies of EIIaE recombinants bearing external deletion (13-15) or linker scanning (LS) mutations (16, 17). In addition to the T1 and T2 pseudo-TATA elements, specific to the EIIaE1 and EIIaE2 promoters, respectively, four upstream elements (A, B, B' and C) are essential for EIIaE1 promoter activity, two of them (A and B) being required for EIIaE2 promoter functions (15 and Fig. 1 for a summary).

As an indispensable step toward the understanding of the mechanism by which the EIa products activate transcription, we have chosen to characterize the cellular factors implicated in the constitutive expression of the Adenovirus type 2 EIIaE TU by in vitro transcription and DNase I footprinting analysis. Using non-infected HeLa cell extracts, optimized for promoters lacking consensus TATA box elements (18) we show that nearly all of the in vivo transcriptional effects of EIIaE promoter mutations altering the critical sequence components could be faithfully reproduced in vitro. These results demonstrate the presence, in the crude extracts, of the essential protein components of the transcription machinery required for specific EIIaE transcription.

In order to map the binding sites of these proteins, DNAse I footprinting analysis has been performed with both nuclear extracts (NE) and semi-purified fractions from whole cell extracts (WCE) of HeLa cells. DNA-binding proteins in the crude NE clearly protect sequences comprised within the

upstream B and C promoter elements against DNAse I digestion and provide weak protection of the T1 element. In addition, the factors recognizing the T1 element have been chromatographically separated from those which bind to the upstream B and C elements.

MATERIAL AND METHODS

Recombinants.

The XbaI linker substitution (LS series) and internal deletion mutants (Δ series) were constructed as described (17). A mutant containing two linker substitutions (LS-7162/-4839) has been obtained by insertion of a synthetic oligonucleotide comprising the EIIaE wild type sequence between -62 and -48 flanked by XbaI linkers into the XbaI site of Δ-7139. In the Δ-129/-87 mutant, the SmaI-XbaI fragment of LS-9787 was replaced with the analogous one from Mp9EII3'-129 (17).

Crude cell extracts and partially purified protein fractions.

HeLa cell nuclear extracts (NE) were prepared as described (19) but the procedure was optimized for efficient *in vitro* transcription (20). Whole cell extracts (WCE) were prepared as described (18). Active transcription factors were partially purified by chromatography of the WCE on an heparin-sepharose column (21). The flow-through material (HFT), containing an essential transcription stimulatory factor (the 43 kD STF, 22), was collected and the adsorbed activity was eluted with 0.6 M KCl, yielding the HO.6 fraction. These proteins were further fractionated (23, 24) by chromatography on a DEAE-matrix and stepwise elution with buffers containing 0.15, 0.20, 0.25 and 1 M KCl, giving the DEO.15, DEO.20, DEO.25 and DE 1M fractions, respectively. Protein concentrations were determined as described in the 1985-86 general handbook Catalog of the Pierce Chemical Company.

In vitro transcription.

In vitro transcription of the EIIaE recombinants (100 to 500 ng of supercoiled DNA per incubation) was carried out in the presence of NE (20), WCE (18) or HO.6 (21). The *in vitro* synthesized RNA was analysed by quantitative S1 nuclease (Appligène, Strasbourg) mapping as previously described (9) and specific transcripts were quantitated by densitometric scanning of the corresponding signals on the autoradiograms.

DNAse I protection.

The standard DNAse I footprinting assay (7) has been used, and modified for experiments with NE (25) and for experiments with DE fractions (23), except that the unlabelled carrier DNA present in each assay was poly dI:dC (Sigma) (50 ng per 20 µl binding reaction). The amount of DNAse I (Sigma, 1550 Kunitz units/mg protein) per assay was empirically determined to yield "balanced" digestion patterns in the presence or absence of varied concentrations of proteins corresponding to the NE, DEO.15, or DEO.25 fractions. Generally, the 5 min. digestion reaction was performed in the presence of 20 ng DNase for naked DNA, 40 to 100 ng for the DE fraction and 200 to 600 ng for the NE.

Preparation of the labelled DNA templates for footprinting.

Selected LS and Δ recombinants were linearized by SmaI (position -250 on the EIIaE promoter) or PvuII (position +62) digestion and treated with calf intestine phosphatase. After 5' end-labelling with [γ-^{32}P]ATP and T4 polynucleotide kinase, the DNA ($\sim$ 5-10,000 cpm per ng) was recut with the complementary enzyme (PvuII or SmaI, respectively) and the labelled EIIaE fragments were electrophoretically purified. The footprints generated on the SmaI-labelled fragments correspond to the non-coding strand; those obtained on the PvuII-labelled fragments are referred to as the coding (i.e. trans-

cribed) strand. The G+A sequence pattern (26) was verified for each preparation and the integrity of the fragments was systematically tested by denaturing polyacrylamide gel electrophoresis. Fragments were stored in TE buffer (50 mM Tris HCl pH 7.9, EDTA 1 mM) at -20°C for up to 3 weeks.

RESULTS

Comparative analysis of in vivo and in vitro template efficiencies of EIIaE recombinants.

Reconstitution of EIIaE transcriptional activity *in vitro*, has been obtained with three types of extracts : crude nuclear (NE) and whole cell extracts (WCE) from HeLa cells and a WCE-derived fraction corresponding to the 0.6 M KCl eluate from an heparin-sepharose column (HO.6, see Materials and Methods). This fraction contains all of the factors required for RNA polymerase B-dependent transcription (21), except for the 43 kD factor, which is crucial for the formation of the preinitiation complex (22, 27) and is therefore added to the reaction mixtures, together with the HO.6 fraction. The principal EIIaE linker scanning (LS) and internal deletion (Δ)-recombinants used for *in vitro* transcription analysis are schematically

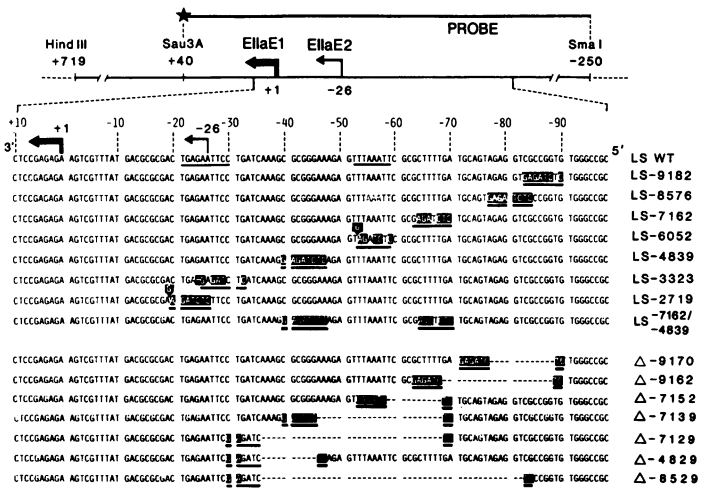

Fig. 2 : Nucleotide sequence of the EIIaE LS and Δ recombinants.
A schematic representation of the SmaI (-250)-HindIII (+719) fragment of the EIIaE TU is given with the relevant nucleotide sequence (non-coding strand) of the LS and Δ series of recombinants (see Material and Methods). The probe, 5' [^{32}P]-labelled at the Sau3A site, used for S1 nuclease analysis is indicated. The TATA-like T1 and T2 elements are underlined in the LS WT sequence. For the LS and Δ series, the XbaI linker sequence is underlined and those nucleotides which have been altered from the wild type sequence are indicated by shaded boxes.

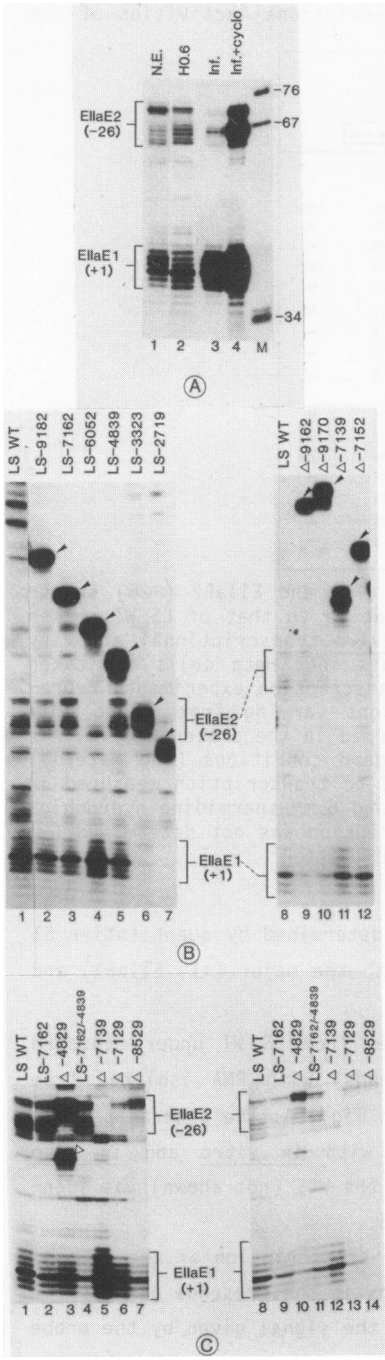

Fig. 3 : Quantitative S1 nuclease analysis of RNA transcribed in vitro from the LS- and Δ-recombinants.

A comparative analysis of the EIIaE transcriptional pattern is shown in panel A. The in vitro transcripts obtained under standard incubation conditions (Material and Methods) from LS WT in the presence of NE (lane 1) or HO.6 (lane 2) are compared to the EIIaE transcripts accumulated in the cytoplasm of adenovirus-infected HeLa cells (12) 5 h post-infection (lane 3) or 6 h post-infection in the presence of 25 µg/ml of cycloheximide added after 2 h of infection (lane 4). Lane M corresponds to ^{32}P-labelled MspI fragments of pBR322, used as size markers. The relative in vitro transcription efficiencies of representative sets of LS-and Δ-mutants is shown in panels B and C. The various templates are incubated in the presence of HO.6 either under standard conditions (panel B, lanes 1-12 and panel C, lanes 1-7) or in the presence of spermidine (panel C, lanes 8-14), as described in Materials and Methods and the legend to Table 1. The probe used to quantitate the EIIaE transcripts is shown in Table 1. The probe fragments protected by specific transcripts are bracketted and correspond to those bands which have been quantitated in Table 1. The arrowheads designate bands corresponding to transcripts initiated at or upstream of the position of the linker substitution. The open arrowhead (panel C, lane 3) points to transcripts initiated around position -19 (see Results).

Table 1 : Relative in vitro and in vivo transcriptional activities of the EIIaE promoter.

	Transcription efficiency of EIIa recombinants (% of WT)			
	In vitro		In vivo	
	+1	-26	+1	-26
LS WT	100	100	100	100
LS-9182	80	70	30	26
LS-8576	100	76	46	45
LS-7162	68	100	26	100
LS-6052	120	20	105	50
LS-4839	80	90	8	30
LS-3323	3	-	3	-
LS-2719	1,5	-	5	-
LS$^{-7162/}_{-4839}$	40	100	4	50
Δ-9170	10	30	12	10
Δ-9162	10	40	2	2
Δ-7152	120	15	106	15
Δ-7139	120(60)	15	35	15
Δ-7129	50(20)	-	19	-
Δ-4829	100(20)	-	18	-
Δ-8529	50(10)	-	3	-

The template efficiency for both EIIaE1 (+1) and EIIaE2 (-26) transcription of each recombinant is expressed relative to that of LS WT, taken as 100%. The values corresponding to the in vivo transcriptional activity measured after transfection of the recombinants into HeLa cells are taken from ref. 17. The results of the in vitro transcription experiments represent the mean values (with standard deviations varying from 10 to 20%) obtained from 5 to 10 independent assays performed in the presence of either NE, WCE or HO.6 under the corresponding standard conditions (see Material and Methods). Values in parentheses correspond to transcription measured in the presence of 1 mM (instead of 5 mM) $MgCl_2$, and 6 mM spermidine hydrochloride. For some recombinants the EIIaE2 transcription was not determined (-) due to interference with the probe homology end-point.

depicted in Figure 2. Template efficiency was determined by quantitative S1 nuclease mapping of the RNA initiated from both the major (+1, EIIaE1) and minor (-26, EIIaE2) startsites.

A comparative analysis of RNA synthesized from LS WT under standard incubation conditions (see Material and Methods) with RNA isolated from adenovirus- infected HeLa cells is presented in Fig. 3A. The general pattern of S1 nuclease-resistant fragments obtained with in vitro and in vivo synthesized RNA is similar. That obtained with the WCE (not shown) was identical to that generated in the presence of HO.6.

A representative analysis of the in vitro transcription of the LS- and Δ-recombinants, tested under standard conditions in the presence of the HO.6 fraction is shown in Fig. 3B. The intensity of the signal given by the probe homology end-point (arrowheads, see legend to Fig. 3) can be taken here as

an internal reference from one mutant to the other. The quantitative data for 5-10 similar assays, performed with different preparations of template DNA and extracts (WCE, NE, HO.6), is presented in Table 1, where they are compared with the corresponding in vivo template activities (17).

1) The T1 and T2 promoter elements are essential for efficient in vitro transcription.

Recombinants with mutations in the TATA-like T1 element for EIIaE1 (LS-3323 and LS-2719) are very poorly transcribed in vitro (1.5-3.0% of WT levels, Fig. 3B, lanes 6 and 7), in agreement with their low template efficiencies in vivo. Similarly, the in vivo phenotypes of the EIIaE2 TATA- like mutants (LS-6052, Δ-7152, and Δ-7139) are well reproduced in the in vitro assay (Fig. 3B, lanes 4, 11 and 12; Fig. 3C, lanes 5 and 12). Note that the signals which appear in the -26 region for recombinants Δ-7129 and Δ-8529 (Fig. 3C, lanes 6, 7, 13 and 14) essentially correspond to non- specific RNA initiated further upstream and revealed around position -29, which is the end-point of homology between the probe and the synthesized RNA. Thus, the reproduction in the in vitro system of the in vivo effects of the TATA-like mutations strongly suggests that the active transcription factors which recognize the T1 and the T2 EIIaE promoter elements are present in the HeLa cell extracts.

2) Maximal in vitro promoter activity requires elements A and B but not element B'.

Deletion of upstream region B in the Δ-9170 mutant severely impairs in vitro EIIaE1 promoter activity, thereby indicating that the relevant upstream transcription factors are also present in the various extracts (Table 1 and Fig. 3B, lane 10). However, the sequence requirement in region B is apparently less stringent in vitro than in vivo, as indicated by the weaker transcriptional effects of the punctual mutations in LS-9182 and LS-8576 (Table 1 and Fig 3B, lane 2, and unpublished data). The mutant, Δ-9162, which contains a deletion of the EIIaE1-specific B' element in addition to the B element is transcribed in vitro with the same efficiency as Δ-9170 (Fig. 3B, lane 9 and 10), implying that element B' is dispensable for in vitro transcription. The observation that the LS-7162 mutation (which defines element B') only slightly affects EIIaE1 in vitro transcription (Fig. 3B, lanes 3 and 3C, lane 2) supports this conclusion. In this respect, it is interesting to note that the amount of transcripts initiated at the EIIaE2 site, relative to those originating from the EIIaE1 site on the WT constructs, is about 10-fold greater in the in vitro assay than in infected

cells (Fig. 3A, compare lanes 1 and 2 with lane 3). Furthermore, the relative ratio of EIIaE1 versus EIIaE2 transcripts synthesized in vitro reflects more closely the pattern of viral RNA accumulated in infected cells in the presence of the protein synthesis inhibitor, cycloheximide (Fig. 3A, lane 4). This suggests that the factor(s) involved in startsite selection is absent or inactive in the cellular extracts used in the in vitro transcription studies or in cycloheximide-treated cells.

In standard in vitro incubation conditions, upstream element A is very poorly recognized, since mutations, LS-4839, Δ-4829, which are deleterious to EIIaE in vivo transcription, do not dramatically alter its in vitro activity (Fig. 3B, lane 5 and 3C, lane 3). The new initiation site appearing around position -19 in the case of Δ-4829 (open arrowhead in Fig. 3C, lane 3) is most likely related to the shift of the T2 element, located 30 bp upstream, in this Δ-mutant. A double mutation (LS-7162/-4839 mutant), altering both regions A and B', has an in vitro effect on EIIaE1 transcription which is that expected from simple addition of the separate effects of each LS mutation (Table 1 and Fig 3C, lanes 4 and 11), indicating that the two elements are functionally independent. In addition, the existence of a negative regulatory element positioned between -62 and -48 is revealed by the 2 to 3-fold higher EIIaE1 in vitro promoter activity of Δ-7152 compared to LS-7162 and Δ-7139 compared to LS-7162/-4839 (Table 1). A repressor effect of about 10-fold is also observed in the transient in vivo expression assay (Zajchowski et al., in preparation and see Table 1). It is likely that at least part of this effect is due to the deletion of the EIIaE2 TATA-like element, leading to increased transcription from EIIaE1 in Δ-7152 and Δ-7139, as a consequence of the transfer of factors from one promoter (EIIaE2) to the other (EIIaE1). However, since transcription from the EIIaE2 promoter is generally lower than that from the EIIaE1 promoter on the WT template (especially in the in vivo assay; see Fig. 3A and ref. 17), such a shift of transcription factors is unlikely to account by itself for the 3-fold (in vitro) or 10-fold (in vivo) increased EIIaE1 promoter activity on these Δ-mutants.

It has been shown that varying the incubation conditions for in vitro transcription by the addition of spermidine magnifies the effects of mutations within the SV40 enhancer sequences (20). While the addition of spermidine has no marked effect upon the transcription of the LS mutants, relative to the LS WT under the same conditions, the relative promoter activities of Δ-4829, Δ-7139, Δ-7129 and Δ-8529 are drastically reduced, in better agreement with the in vivo promoter strengths of these mutants where element A is

altered (compare lanes 10, 12-14 to lanes 3, 5-7 in Fig. 3C and the values in parentheses in Table 1).

Therefore, with the exception of the sequences defining element B', we have demonstrated here that the elements required for efficient in vivo transcription of the EIIaE TU are also critical for in vitro template activity. The weak contribution of element C to the EIIaE in vivo promoter activity (15) has not been further investigated in the present in vitro study. However, previous experiments by Elkaim et al. (13) could not reveal any significant effect on in vitro transcription efficiency of deletions removing this upstream element.

DNA protection experiments

1) Three binding domains are revealed in the EIIaE promoter by footprinting experiments with crude extracts and partially purified fractions.

To directly visualize the interaction of specific transcription factors with promoter elements, DNAse I protection experiments have been performed on the EIIaE promoter fragment (Fig. 4A, lanes 2-4). NE proteins from uninfected HeLa cells protect two major regions on the non-coding strand : sequences between -82 and -68 and between -146 and -128, comprising the upstream elements B and C, respectively. A weaker protection is apparent between -105 and -88 and a slight decrease in cleavage sites between -36 and -19 corresponding to the EIIaE1-specific T1 element is reproducibly detected. An additional protected region is observed around +30, but its location near the end of the probe fragment raises doubts as to its significance. Sites hypersensitive to DNAse I cleavage are noted at positions -84 and -50.

In an attempt to improve the DNA protection patterns, and further characterize the factors involved, the crude extracts were partially purified, as previously described (see Material and Methods, 23 and 24). The DE0.15 and DE0.25 fractions, which have been shown to contain the adenovirus major late promoter (MLP) upstream and TATA-box factors, respectively (23), were able to promote specific transcription from the EIIaE1 startsite when combined with purified RNA polymerase B and the fraction containing the 43 kD stimulatory factor (data not shown). This combination of fractions efficiently reconstitutes transcription from the Ad2 MLP (24), but is 5 to 10-fold less active than the crude extracts in catalyzing EIIaE in vitro transcription, even after addition of the complementary DE0.20 and DE 1M fractions (data not shown).

In footprinting analyses, the DE0.15 fraction generates the same pro-

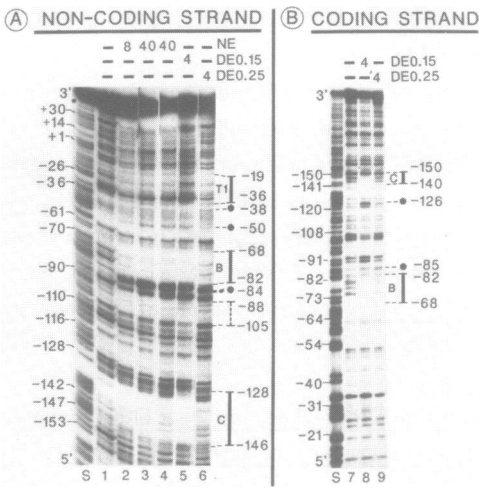

Fig. 4 : DNAse I footprinting on the wild type EIIaE promoter region.
A comparative analysis of the DNAse I protection provided by the NE (lanes 2-4), DE0.15 (lanes 5 and 8) and DE0.25 (lanes 6 and 9) fractions is shown on the non-coding (panel A) and coding strand (panel B) of the SmaI-PvuII EIIaE fragment as described in Materials and Methods. The amounts (in µg) of protein used in each assay are indicated on the top. The profile in lane 4 has been obtained with a different NE preparation from that in lanes 2 and 3. The DNAse I digestion patterns of the naked probes are shown in lanes 1 and 7. The corresponding G+A sequence ladders are given in lanes labelled S; the numbering on the left indicates the nucleotide positions relative to the EIIaE1 (+1) startsite. The major effects of the nucleo-protein interactions are schematically summarized on the right of each panel : areas most strongly protected are marked by solid bars, those more weakly protected by dotted bars, and DNAse hypersensitive sites by heavy dots. The limits or positions of these regions on the EIIaE fragment are indicated. T1, B and C refer to the promoter elements spanning the major protected sequences.

tection pattern as the NE on the upstream elements of the EIIaE non- coding strand (Fig. 4A, compare lane 5 with 2 to 4; only optimal protein concentrations are shown for the DE fractions). By contrast, proteins in the DE0.25 fraction confer significant protection against DNAse I digestion of the T1 domain between -36 and -19 and reproducibly augment the cleavage at position -38, but fail to protect any portion of the upstream element C and the region between -105 and -88 (Fig. 4A, lane 6). The partial protection exerted over the -82/-68 sequences suggests that the DE0.25 fraction is contaminated by some of the factors present in the DE0.15 fraction.

The footprint pattern was also examined on the coding strand of the EIIaE fragment (Fig. 4B). The DE0.15 fraction strongly protects the sequence

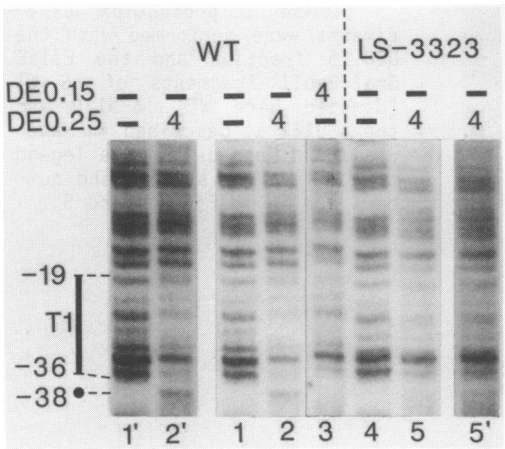

Fig. 5 : DNAse I footprinting (non-coding strand) on the T1 promoter element.

The nucleoprotein interactions were analysed (see Materials and Methods, and legend to Fig. 4) with the DE0.15 and DE0.25 fractions on the LS WT (denoted here WT) and LS-3323 recombinants described in Fig. 2. Lanes 1', 2' and 5' correspond to longer exposures of lanes 1, 2 and 5, respectively. The sample in lane 5 being slightly overdigested, lane 4 compares more directly to lane 5' than to lane 5. The intensity of invariable bands (above position -19) in lane 4 is indeed closer to that in lane 5'. The protected region and the hypersensitive site at -38 are marked on the left.

between -82 and -68 (element B) and also footprints on upstream element C, between -150 and -140, with the generation of a DNAse I hypersensitive site at -126. This fraction and the crude NE give identical results (data not shown). No protection of the T1 element is visible after incubation of the DE0.25 fraction with this strand, even though a weak protection of element B is apparent (Fig. 4B, lane 9). The reasons for the lack of T1 footprint on the coding strand are still unclear, but could reflect a lower stability of the complex on this strand. Further enrichment of the corresponding factor should improve the protection pattern.

The DEAE-matrix chromatographic step, therefore, results in the separation of a factor which interacts with sequences corresponding to the T1 element (in the DE0.25 fraction) from those which recognize the B and C upstream elements (in the DE0.15 fraction). Neither the DE fractions nor the NE protect the upstream A and B' elements from DNAse I digestion, although a DNAse I-hypersensitive site at the 5' border of element A (position -50) is reproducibly induced (Fig. 4A).

2) Analysis of EIIaE promoter mutants confirms the specificity of these interactions.

The specificity of protein binding on the EIIaE promoter has been verified by examining the effect of mutations, within or around the protected segments, on the footprints. These analyses have been performed with the DE fractions, which give equivalent or better protection patterns than the NE.

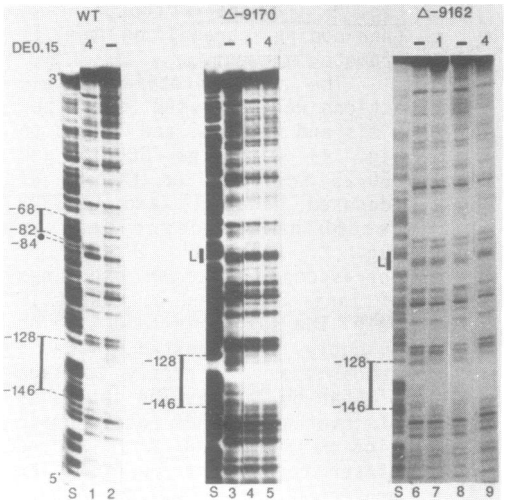

Fig. 6 : Effect on the DNAse I footprint (non coding strand) of recombinants which delete upstream elements B and B'.

DNAse I protection experiments were performed with the DE0.15 fraction and the EIIaE SmaI-PvuII fragments of LS WT (denoted here WT), Δ-9170 and the Δ-9162 as described in Materials and Methods and the legend to Fig. 4. The symbols and numbers are as in Figs. 4 and 5.

Alteration of the T1 element by the LS-3323 mutation (Fig. 5) or by LS-2719 (data not shown) diminishes the protection of the -36/-19 region by the DE0.25 proteins and eliminates the DNAse I hypersensitive site at position -38 (Fig. 5, compare lanes 1' and 2' or 1 and 2 for the WT template with lanes 4 and 5 or 5' for LS-3323). As discussed for Fig. 4, the DE0.15 proteins do not provide significant protection to the T1 region (Fig. 5, compare lanes 1 and 3). For the LS-3323 recombinant, no modification in the protection pattern for other promoter regions was observed with neither of these DE fractions (not shown), indicating that the mutation affects only T1 element recognition.

Since the upstream internal deletion mutations in Δ-9170 and Δ-9162 produced the most dramatic effects on in vitro template activity of all of the region B mutants tested, we verified that protein binding was also abolished. The mutations prevent the binding of element B-associated proteins on both strands (Fig. 6 and data not shown). In contrast, the punctual mutations in the 5' border of element B (LS-9182, Fig. 7B, lanes 3 and 4, and LS-8576, data not shown) do not significantly affect the protection of this region, as expected from the nearly wild type in vitro transcriptional activities of these mutants (see Table 1). The LS-7162, Δ-7152 and Δ-7129 mutations which alter 3 nucleotides at the 3' border of the protected B region (positions -68 through -70), do not affect the extent of this protection but reproducibly reduce the intensity of the footprint on both DNA strands (see Fig. 7). Even if in some cases the protection appears unchanged on the

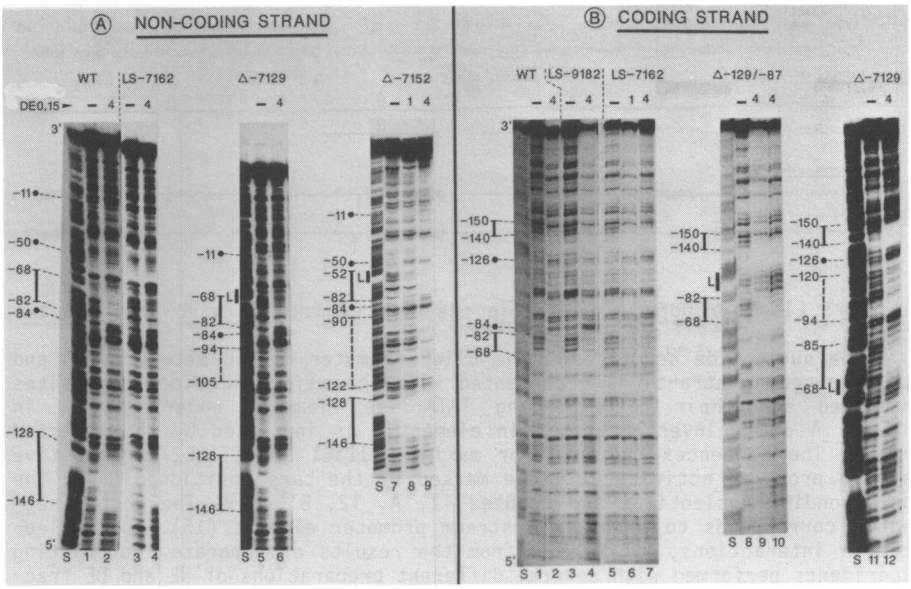

Fig. 7 : DNAse I footprinting with the DE fractions on selected EIIaE upstream promoter mutants.

The nucleoprotein interactions were analysed (see Materials and Methods and legend to Fig. 4) with the DE0.25 or DE0.15 fractions and the various EIIaE recombinants as indicated at the top (WT corresponds to the LS WT recombinant described in Fig. 2). Lanes 9 and 10 (panel B) correspond to samples incubated in the presence of equal amounts of the indicated DE fraction, but digested with different concentrations of DNAse I (80 ng for lane 9 and 60 ng for lane 10). The position of the XbaI linker sequence is indicated next to the G+A sequence ladder (S) of the Δ-mutants by the solid block (L). The major protections and hypersensitive sites are denoted on the left of each autoradiogram. Note that the WT sequence numbering is retained even for the mutants which delete significant portions of it. Hence, in the case of Δ-7129 where the 3' limit of the footprint is situated within the linker sequence, position -68 actually corresponds to the C at position -35 (according to the sequence representation in Fig. 2).

mutant compared to the WT template (compare in Fig. 7A, lanes 2 and 9), the footprint always appears at lower protein concentrations on the WT template (data not shown).

The protection of upstream element C remains unaffected in all of the mutants described above, suggesting that there is no essential physical interaction between the factors binding to the far upstream element and those recognizing the sequences further downstream. This conclusion is supported by the analysis of the Δ-129/-87 deletion mutant, where the spacing between elements B and C has been altered : no footprint is generated over

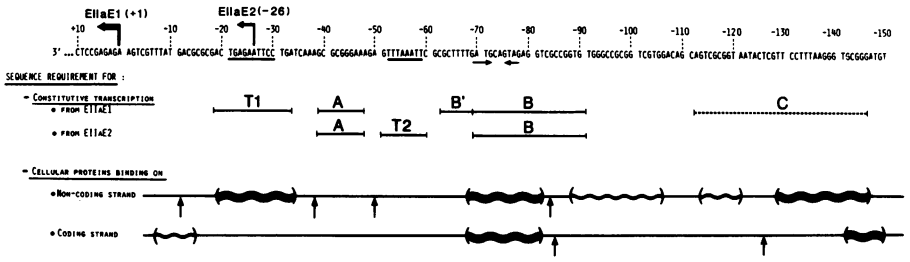

Fig. 8 : EIIaE promoter organization as deduced from in vivo and in vitro studies.

The nucleotide sequence of the EIIaE promoter region between -150 and +10 (non-coding strand) is represented with the major and minor startsites indicated and their corresponding TATA-like elements underlined as in Fig. 2. A small inverted repeat in element B is indicated by the inverted arrows. The sequences required for maximal EIIaE1 and EIIaE2 constitutive in vivo promoter activity (17) are marked by the bars positioned under the corresponding nucleotides and denoted T1, A, T2, B' or B. The dotted segment C corresponds to a weaker upstream promoter element (15). The nucleoprotein interactions, as deduced from the results of separate footprinting experiments performed with several different preparations of NE and DE fractions, are schematically represented by the bracketted wavy symbols, with the heavy and thin lines depicting strong and weak interactions, respectively. DNAse I hypersensitive sites are noted by the vertical arrows.

the XbaI linker between -129 and -87, while protection of the upstream B and C elements is unchanged (Fig. 7B, lanes 8-10).

Hence, DNA fragments from EIIaE promoter mutants, which have reduced in vivo and in vitro template efficiencies, show no or weakened footprints on the mutated regions. This suggests, therefore, a direct correlation between specific protein binding to promoter elements T1 and B and EIIaE transcriptional activity.

DISCUSSION

The complex promoter structure of the adenovirus EIIaE TU has been analysed by in vitro transcription and DNAse I protection studies, using crude extracts and partially purified fractions from uninfected HeLa cells. By comparing the template efficiencies of a series of linker scanning and internal deletion mutants, we demonstrate that faithful and efficient transcription is dependent upon the promoter region corresponding to the EIIaE1 (T1) and EIIaE2 (T2) TATA-like sequences, and to upstream sequences A and B. By footprinting analyses, we show that promoter elements T1, B and C bind specific cellular factors, with the T1 factor being chromatographically separable from the B and C factors (see Fig. 8).

The in vivo transcriptional phenotypes of the templates, which have mutations in the T1 or T2 elements or deletions of the upstream B factor binding site, are perfectly reproduced in the in vitro transcription systems with crude WCE and NE or with the HO.6 fraction. While the requirement of upstream promoter elements for efficient in vitro transcription has been largely documented (3, 6, 28, 29), the role of non-consensus pseudo- TATA sequences has been established in only a few cases. Our results clearly emphasize the importance of the adenovirus EIIaE TATA- like elements in determining the transcriptional efficiency of the EIIaE TU. In similar studies, a transcription-controlling function has been demonstrated for the TATA-like sequences in the SV40 late (30) and the adenovirus IVa2 promoters (18).

Mutations which alter the adenovirus EIIaE upstream region B' (i.e. LS-7162) have very little effect upon promoter strength, indicating (i) that the putative corresponding factor, different from that which binds element B, is absent, inactive, limiting, or unbalanced with respect to the other factors in these extracts or (ii) that the mutation modifies a secondary or chromatin structure of the template DNA which is not formed in vitro. In this respect, it is noteworthy that the relative amounts of LS WT-coded transcripts from the EIIaE1 and EIIaE2 intiation sites in vitro resemble more closely the in vivo transcription pattern of the LS-7162 mutant, where the amount of transcripts from both startsites is nearly equal (17). This would be expected if the factor and/or active conformation associated with region B' which are implicated in the control of startsite selection are not functional in the in vitro system. Support for this interpretation comes from the experiments with cycloheximide. This treatment alters the relative in vivo startsite utilisation, effectively increasing the ratio of EIIaE2 to EIIaE1 transcripts and suggests the involvement of short-lived proteins in this process.

The role of element A in EIIaE transcription could not be observed under the standard incubation conditions, but was specifically revealed upon addition of spermidine to the in vitro assay. The mechanism by which spermidine influences transcription is unknown, but it has been shown to stimulate the transcriptional activity of purified RNA polymerase B (31) and to augment the transcriptional effects of punctual mutations in the SV40 enhancer in NE-driven transcriptions (20).

As an initial step towards the characterization of the DNA-binding proteins implicated in the transcription of EIIaE, DNAse I protection studies were performed with crude extracts and semi-purified fractions from

WCE. From these studies, we conclude that a factor, which specifically binds the T1 element of the EIIaE promoter is enriched in the DE0.25 fraction, where the TATA-box factor responsible for the activity of a number of polymerase B promoters, including those with consensus TATA sequences, is also found (Tamura et al., in preparation). No protection in the region of the T2 element has been observed in any of the assays, but it cannot be excluded that the low abundance of DNAse I cleavage sites in this region prevents its visualization for purely technical reasons. The DNAse I hypersensitive site at position -50 is probably not related to a protein interaction with the T2 element (but rather with the A element), since deletion of the element, as in the Δ-7152 recombinant, does not eliminate the hypersensitive site even though EIIaE2 in vitro promoter activity is severely reduced. To determine whether the factors which recognize the T1 and T2 TATA-like elements and the consensus TATA sequence are identical, in vitro competition studies using the more purified TATA-box factor preparations characterized by Tamura et al. (in preparation) are in progress.

In the DE0.15 fraction, the upstream factor B is enriched about 10-fold compared to the NE in its capacity to protect the EIIaE sequences between -82 and -68 from DNAse I digestion (as could be deduced from comparison in Fig. 4A of lanes 3 and 4 with 5). This footprint is abolished upon deletion of the pertinent sequences, but modifications in the 5' region of the footprint (LS-8576 and LS-9182), have no effects upon the protection pattern, explaining why they do not significantly perturb the in vitro transcriptional activity.

By contrast, mutations in the 3' border of the protected B region (LS-7162, Δ-7152 and Δ-7129) decrease the affinity of the protein for the B element. This may account for the slightly reduced in vitro transcriptional activity of LS-7162. The higher template activity of Δ-7152 and Δ-7129 is then most likely explained by the simultaneous deletion of a negative regulatory element, located between -62 and -48 (see Results), together with part of the B element.

The recognition sequence for upstream factor B resembles none of the consensus binding sites for the well-characterized Sp1 protein (32, 5), MLP upstream factor (23, 33, 34), or CCAAT protein (35, 36), implying that it may belong to another family of upstream sequence-interacting transcriptional factors. Results from competition experiments performed between the MLP and EIIaE in in vitro transcription and footprinting analyses have also indicated that the upstream factor of the MLP is different from the upstream factor B of the EIIaE promoter (23, 37). Sequences homologous to upstream

region B (between -78 and -69) can, however, be found in other adenovirus early gene promoters, including the enhancer region of EIV (between -173 and -158 and -149 and -132, 38), the upstream sequences of EIII (between -96 and -88, 39), and two upstream regions of EIa (between -405 and -396 and -268 and -259). Further purification and characterization of protein factor B, as well as the appropriate transcription and binding competition studies, should determine its involvement in the control of constitutive transcription from this and other EIa-responsive TU.

The DE0.15 fraction also contains proteins which bind with high affinity to far upstream sequences (-150 to -128) within the C element. No direct evidence exists for a role for this DNA-binding protein in EIIaE transcriptional activity in vitro (13). However, a minor influence of the sequences between -146 and -111 in in vivo constitutive promoter strength has been reported (15) and we have recently demonstrated an involvement for these sequences in EIa-mediated stimulation of EIIaE transcription (Zajchowski et al., in preparation). Within this zone, there is another, more weakly protected region, between sequences -120 and -115 (see Fig. 8). It cannot be excluded therefore that the in vivo transcriptional effects of element C are also, or instead, related to factor binding in this region.

Kovesdi et al. (40, 41) have suggested the involvement of a specific cellular protein factor in the EIa-mediated activation of the EIIaE promoter. This factor, whose binding activity is greatly increased in the presence of the EIa products, recognizes a sequence element between position -74 and -33 (40). In agreement with this observation, our experiments with extracts from uninfected HeLa cells failed to detect any protection in this region comprising the elements T2, A and B'. It is likely therefore that these elements represent potential targets for specific transcription factors activated by the EIa products in adenovirus (Ad)-infected cells. That the factor recognizing element B is also implicated in the EIa-mediated response is suggested by the in vivo exonuclease mapping experiments (41), which reveal efficient protein binding to sequences downstream from -85 (thus comprising the element B), but only in cells infected with wild type adenovirus. It is surprising, however, that these authors did not detect the corresponding factor in their subsequent in vitro studies (40) with neither Ad-infected nor uninfected cell extracts. The reasons for the discrepancy of these in vitro results and ours are unclear but could be related to differences in the preparation of cell extracts, since the extraction procedure was not optimized for in vitro transcription efficiency in the case of Kovesdi et al. (40). Furthermore, in contrast to the results of Kovesdi et

al. (40) a recent report by SivaRaman et al. (42) demonstrates the presence of this B factor, with the same specific DNA-binding capacity in both uninfected and Ad-infected HeLa cell extracts. It is therefore unlikely that the EIa-mediated transcriptional activation is exerted through differential binding of this factor. However, using a transient in vivo expression assay, Jalinot and Kédinger (43) and Zajchowski et al. (in preparation) have recently demonstrated that the B region is involved in the EIa-responsiveness of the EIIa promoter. This suggests therefore, as previously proposed (44), that the EIa products may act by catalysing productive interactions between the different proteins of the transcription complex, rather than by modulating their DNA-binding activity. The precise knowledge of the sequence components and the proteins implicated in the constitutive function of the EIIaE promoter and, ultimately, the purification and comparison of the transcription complexes from uninfected and Ad-infected cells, will help to clarify this question.

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