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Far upstream sequences are required for efficient transcription from the adenovirus-2 E1A transcription unit

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ABSTRACT

We have investigated the requirement for sequences located upstream from the TATA box for efficient transcription from the Adenovirus-2 (Ad2) E1A promoter. A series of deletions located within the E1A promoter upstream sequences were introduced into recombinants which contain or do not contain the E1A structural sequences. The amount of E1A-specific RNA produced after transfection into HeLa cells was determined by quantitative S1 nuclease analysis. We demonstrate that sequences located more than 231 bp upstream from the E1A capsite are required for efficient transcription from the E1A promoter. However, the requirement for these stimulatory sequences is less pronounced in recombinants which contain the E1A structural sequences than in those in which these sequences have been deleted. We demonstrate also that these Ad2 stimulatory sequences activate transcription in cis when inserted upstream from the heterologous -34 to +33 Ad2 major late promoter (Ad2MLP) element which is otherwise inactive when transfected into HeLa cells. These results suggest that the 270 bp Ad2 left-terminal segment contains an enhancer-like element.

INTRODUCTION

The Adenovirus-2 E1A transcription unit, located in the left-terminal region of the viral genome, is the first to be transcribed during infection (fig. 1A). Three overlapping messenger RNAs, two produced immediately after infection (12S and 13S) and one produced only during the late phase (9S) (1-3), are initiated from a main capsite located 498 bp from the Ad2 left-end (4). E1A products are essential for efficient expression of the other early transcription units during infection (5-10). In addition the cloned E1A transcription unit is efficiently transcribed when transfected into HeLa cells, whereas the other early transcription units are poorly expressed, unless cotransfected with an E1A recombinant (11-15). These observations raise the possibility that the E1A promoter region may contain a stimulatory element that renders it particularly efficient. In this paper we show that sequences located more than 231 bp upstream from the E1A capsite are required for efficient expression of the E1A transcription unit, and that these sequences can activate transcription from a heterologous promoter.

MATERIALS AND METHODS

Enzymes

Restriction enzymes were purchased from BRL, Biolabs and Boehringer. S1 nuclease was from Miles. T4 DNA ligase, DNA polymerase I and T4 polynucleotide kinase were obtained from Biolabs.

Construction of recombinants pEIASV, pEIA$^-$ and pEIA-A34

pEIASV (Fig. 1B) contains the left-terminal HpaI (0-4.5 m.u.) fragment of Ad2 (Fig. 1A) linked to the 135 bp SV40 HpaI (2604)-BamHI(2469) (16) fragment containing the SV40 polyadenylation signals. These fragments have been inserted between the repaired EcoRI site (regenerating an EcoRI site) and the BamHI site of pBR322. pEIA$^-$ (Fig. 1B) was derived from pEIASV as follows. The EcoRI-Sau3A fragment of pEIASV was cloned between the EcoRI and the BamHI sites of M13mp8. The EcoRI-HindIII fragment of the resulting mp8 derivative was then inserted between the EcoRI and the HpaI sites of pEIASV. pEIA-A34 (Fig. 4A) contains the EcoRI-PvuII fragment of pEIASV (Fig. 1B) cloned between the EcoRI and the SstI site of pSVA34 (17, see also 18), located 63 nucleotides upstream from the Ad2 major late (Ad2ML) capsite. pSVA34 contains the -34 to +33 fragment of the Ad2ML promoter (Ad2MLP) linked to the SV40 coding region (5227-2533) (16).

Transfection of HeLa cells.

HeLa cells (20-40 % confluence) in monolayer were transfected by the calcium phosphate technique as described (19) with 20 μg of recombinant DNA per 10-cm Petri dish and 2 μg of the polyoma-β-globin plasmid pβ(244+)β (20) as an internal control. After 48 hr, cytoplasmic RNA was purified as described (17).

Analysis of RNA by quantitative S1 nuclease mapping.

Single-stranded DNA fragments, 5'-end labelled with [γ-^{32}P]-ATP (Amersham), were purified by electrophoresis (4°C, 250V) on 8 % acrylamide-bisacrylamide (60:1) gels and eluted from crushed gel bands by filtration through siliconized glass-wool (21). RNA from one 10 cm Petri dish (approximately 50 μg) was dissolved in 10 μl of 10 mM Pipes, pH 6.5, 0.4 M NaCl containing an excess of the single-stranded probe indicated in the figure legends and the globin internal control probe (20), and hybridized at 68°C for 12 h. After digestion with 2000 units of nuclease S1 (2 hours at 25°C in 3 mM $ZnCl_2$, 30 mM NaAc pH4.5, 300 mM NaCl), the resistant DNA fragments were analyzed on 8% acrylamide/8.3 M urea gels (21). Quantitation was achieved by scanning the autoradiograms of several exposures. When applicable, the values were corrected for transcription from the cotransfected globin recombinant and correspond to the average of at least four independent experiments.

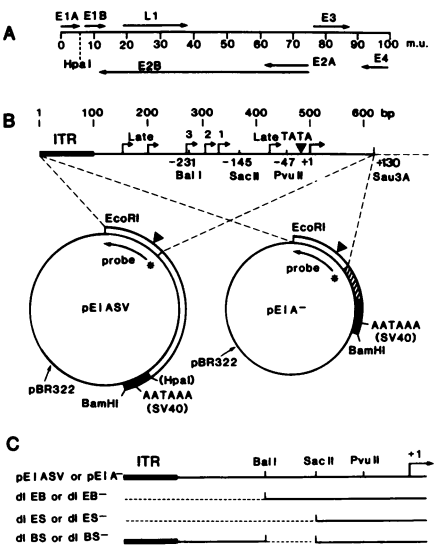

Fig. 1 : A) Schematic representation of the early transcription units of Adenovirus-2 ; m.u. corresponds to map unit. B) Structure of pEIASV and pEIA⁻ (Materials and Methods). The SV40 late polyadenylation signal (AATAAA) is indicated. The cross-hatched segment in pEIA⁻ corresponds to an M13 fragment (see Materials and Methods). On the enlargement of the EcoRI-Sau3A fragment the E1A main capsite is indicated as +1, and the positions of the BalI, SacII, PvuII and Sau3A sites are indicated with respect to the E1A capsite. Positions (in bp) from the left-end of Ad2 are shown above the line. Arrows 1, 2 and 3 designate the E1A early upstream initiation sites (22, 23) discussed in the text. The arrows marked "Late" indicate the initiation sites which appear only late in infection (23). The location of the E1A TATA box is indicated by closed triangles. ITR is the 102 bp inverted terminal repeat (16). The E1A probe, used for quantitative S1 nuclease mapping, 5'-end labeled with $[\gamma\text{-}^{32}P]$-ATP at the Sau3A site (asterisk), is indicated as an arrow inside the plasmid map (see Materials and Methods). C) Schematic representation of the deletion mutants derived from pEIASV and pEIA⁻. The dashed lines show the extent of the deletions. In all three cases the extremities of the deletion were made blunt with the large fragment of E.coli DNA polymerase I before ligation.

RESULTS

A) Characterization of RNA produced in Hela cells transfected with recombinants containing either the entire E1A transcription unit or only the E1A promoter region.

Plasmid pEIASV contains the E1A transcription unit, from the Ad2 left-end to the HpaI site, excepting only the polyA addition site which has been replaced by the SV40 late polyA addition site (see Fig. 1A and B and Materials and Methods). pEIASV was transfected into HeLa cells and E1A RNA was analysed by quantitative S1 nuclease mapping using a single stranded probe 5'-end labelled at the Sau3A site (Fig. 1B). As shown in Fig. 2 the RNA species transcribed from pEIASV (lane 1) were initiated from the same sites as those used during the early phase of Ad2 lytic infection (lane 2). As already reported (4), the main E1A capsite (EIA+1) is located at 498 bp from the Ad2 left end. A series of additional sites situated upstream from the main capsite were also detected and the major ones were mapped (by

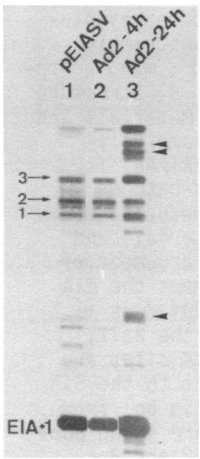

Fig. 2 : Quantitative S1 nuclease analysis (Materials and Methods) of RNA extracted from HeLa cells transfected with pEIASV or infected with Adenovirus-2 using the probe described in legend to Fig. 1B. E1A+1 corresponds to RNA initiated at the E1A main capsite, while 1, 2 and 3 correspond to the upstream minor initiation sites (see fig. 1B). Lane 1 : RNA from pEIASV transfected cells. Lanes 2 and 3 : RNA obtained after infection of HeLa cells with Ad2. Cells were harvested either 4 hours (lane 2) or 24 hours (lane 3) after infection. Arrowheads in lane 3 correspond to initiation sites which appear only late in infection (see fig. 1B).

comparison with a sequence ladder) to positions 338, 314 and 261 from the left-end (sites 1, 2 and 3, in lanes 1 and 2 of figure 2, see also figure 1B), in agreement with the previous results of Osborne, Gaynor and Berk (22, 23). As noted by these authors, additional RNA species initiated mainly further upstream were detected during the late phase of the lytic infection (figure 2, arrowheads in lane 3). The absence of these sites after transfection with pEIASV most likely reflects the inability of this recombinant to replicate (23).

E1A products are known to stimulate the expression of the other Ad2 early transcription units (EIB, E2, E3, E4, see introduction for refs.) and of some cellular genes (24, 25). pEIA$^-$, which lacks the E1A coding sequence (Materials and Methods, figure 1B), was constructed to investigate the possible effect of E1A products on the initiation of transcription from the E1A promoter region. In several transfection experiments similar to that shown in figure 3A, we found that the amount of RNA initiated from the main E1A capsite was approximately 10 times lower in pEIA$^-$ than in pEIASV. Initiation from upstream sites 1, 2 and 3 was also strikingly decreased (figure 3A, lanes 1 and 2).

B) Transcription from the E1A promoter is drastically reduced by deletion of sequences located far upstream from the main capsite

A series of mutants were constructed from pEIASV and pEIA$^-$ by deleting the sequences between the EcoRI and BalI sites (dlEB series), the EcoRI and SacII sites (dlES series) and the BalI and SacII-sites (dlBS series) (figure 1C). These recombinants were transfected into Hela cells and the

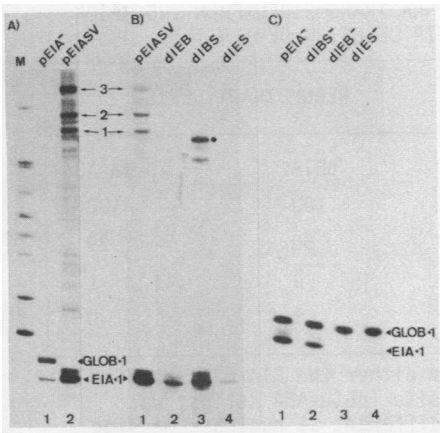

Fig. 3 : Quantitative S1 nuclease analysis of RNA extracted from HeLa cells transfected with pEIASV, pEIA⁻ and their deletion derivatives, using the probe described in Fig. 1B. E1A and 1, 2, 3 are is in legend to Fig. 2. In all the experiments a constant amount of the β-globin recombinant was cotransfected and used as an internal control (Materials and Methods). Glob+1 corresponds to RNA initiated at the β-globin capsite. A) Comparison of pEIA⁻ (lane 1) and pEIASV (lane 2). M is a size marker ([^{32}P]-end-labeled MspI fragments of pBR322). B) Lanes 1 to 4 correspond (as indicated) to the deletion derivatives of pEIASV (Fig. 1C) cotransfected with the β-globin recombinant. Asterisk in lane 3 indicates the endpoint of homology between the RNA transcripts and the probe. C) Lanes 1 to 4 correspond (as indicated) to the deletion derivatives of pEIA⁻ (Fig. 1C) cotransfected with the β-globin recombinant.

RNAs produced were compared by quantitative S1 nuclease analysis to those synthesised from pEIASV and pEIA⁻ (figure 3B and C). Note that the exposure time was approximately 3 times longer for the pEIA⁻ series in panel C than for the corresponding pEIASV series in panel B, whereas the exposure time was the same for pEIASV and pEIA⁻ in panel A. The results of four experiments similar to those shown in panels B and C, quantitated by scanning the autoradiograms, are given in Table 1. Deletion of the EcoRI-BalI fragment from pEIASV (dlEB, panel B, lane 2) completely suppressed the RNA species initiated from the upstream sites and resulted in a 70% decrease in the amount of RNA initiated from the main capsite. Additional deletion extending to the SacII site (dlES, panel B, lane 4) caused a further four-fold decrease in the amount of RNA initiated from the main capsite. In contrast, deletion of the BalI-SacII fragment (dlBS) had very little effect on either initiation from the main capsite, or total initiation from upstream sites as judged from the relative intensity of the band which corresponds to the endpoint of homology of the probe (asterisk, panel B, lane 3).

The EcoRI-BalI deletion in the pEIA⁻ series (dlEB⁻) resulted in a more drastic reduction in RNA initiated from the E1A promoter than the same deletion in pEIASV (20-fold vs 3-fold, table 1 ; compare in fig. 3 lanes 1 and 2 in panel B with lanes 1 and 3 in panel C). When the deletion was further extended to the SacII site (dlES⁻) the same decrease was observed as in the previous series [30% to 7% for the pEIASV series and 5% to 1% for the

Table 1 : RELATIVE AMOUNT (IN PERCENT) OF RNA TRANSCRIBED FROM THE MAJOR CAPSITE OF pEIASV, pEIA$^-$, pEIA-A34 AND THEIR DELETION DERIVATIVES.

	RECOMBINANT SERIES		
	pEIASV	pEIA$^-$	pEIA-A34
Wild-type	100	100	100
Deletion dlBS	80	50	29
Deletion dlEB	33	5	1
Deletion dlES	7	1	0

The relative amount of RNA transcribed from either the E1A main capsite (pEIASV and pEIA$^-$ series) or the Ad2ML capsite (pEIA-A34 series) was estimated by scanning autoradiograms from different S1 nuclease mapping experiments similar to those shown in figures 3 and 4. In each case, the figures are the average of four independant experiments (with different plasmid preparations) which gave similar values (±20%). The values of the pEIA$^-$ and pEIA-A34 series were corrected for transcription from the cotransfected globin recombinant (see text). 100% in the three sets of experiments corresponds to RNA transcribed from pEIASV, pEIA$^-$ and pEIA-A34, respectively. 100% of pEIA$^-$ corresponds to 10% of pEIASV (see text).

pEIA$^-$ series (table 1)]. Deleting the BalI-SacII fragment alone had approximately the same moderate effect in the pEIA$^-$ series (dlBS$^-$, lane 2 panel C and table 1) as in the pEIASV series.

The reference β-globin plasmid, pβ(244$^+$)β (Materials and Methods), was cotransfected with the E1A recombinants in all of the experiments shown in figure 3 in order to correct for possible variations in transfection efficiencies. It is interesting that no transcription initiating at the globin capsite (Glob+1) could be detected when the globin plasmid was cotransfected with the pEIASV series, even though it was readily seen in the presence of the pEIA$^-$ series (compare in fig. 3 lanes 1 and 2 in panel A, and panels B and C). Identical results were obtained in four experiments similar to that shown in figure 3. The absence of the globin transcript was not correlated with the amount of E1A specific RNA, since it was not detected with dlES which produced 15 times less RNA than pEIASV (fig. 3, lanes 1 and 4 in panel B, and table 1). Since the amount of RNA produced from dlES and pEIA$^-$ are very similar (7% and 10% of pEIASV, respectively - see above and table 1), it is clear that the absence of the β-globin transcript is related to the presence of the E1A structural gene.

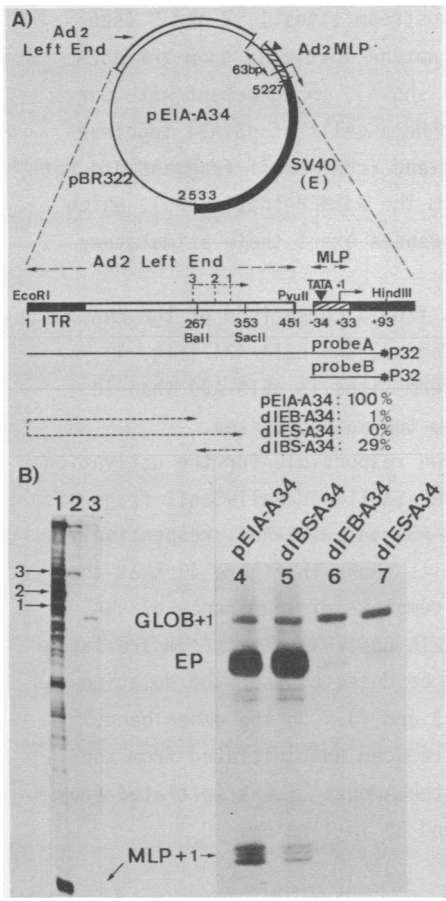

Fig. 4 : A) Structure of pEIA-A34 (Materials and Methods). The -34 to +33 Ad2MLP fragment is linked to the SV40 early coding region (SV40 E) in pSVA34 (17). Positions of the BalI, SacII and PvuII sites are indicated with respect to the Ad2 left-end (position 1, see Fig. 1B). Probes A and B were 5'-end-labeled at the SV40 HindIII site (+93 with respect to the Ad2ML capsite) and extend to the Ad2 left-end (probe A) or position -34 (probe B). dlEB-A34, dlES-A34 and dlBS-A34 are deletion mutants derived from pEIA-A34 (see text). The dashed lines show the extent of the deletions. The figures in percent correspond to the relative amounts of RNA initiated at the major late capsite (MLP+1 in panel B). The two bands present in lanes 2 and 3 correspond to the end-points of homology between probe A and RNA initiated upstream from the BalI and SacII sites, respectively. B) Quantitative S1 nuclease analysis of RNA extracted from Hela cells transfected with pEIA-A34 and its deletion derivatives. Lanes 1, 2 and 3 correspond to pEIA-A34, dlEB-A34 and dlES-A34, respectively, analysed with probe A (no internal control globin recombinant was cotransfected); Lanes 4 to 7 correspond to pEIA-A34 and its deletion derivatives, as indicated, analysed with probe B (the β-globin recombinant was cotransfected). Arrows 1, 2 and 3 and Glob+1 are as in legend to figures 1, 2 and 3. MLP+1, see above. EP corresponds to the endpoint of homology between the RNA transcripts and probe B.

C) Stimulation of transcription from an heterologous promoter by the E1A far upstream sequences.

pEIA-A34 (figure 4A and Materials and Methods) was constructed to test the possibility that E1A far upstream sequences can stimulate transcription from heterologous promoter elements. The EcoRI-PvuII fragment of the Ad2 left-end was inserted into recombinant pSVA34 (17, see also 18) upstream from a segment of the Ad2MLP (-34 to +33) (Fig. 4A). Two probes (A and B, figure 4A) were used for S1 nuclease analysis of RNA initiated from pEIA-A34. As shown in figure 4B (lane 1), probe A detected RNA initiated

both at the Ad2ML capsite and at the E1A upstream sites 1, 2 and 3 (see above), whereas under the same conditions no RNA initiated from the Ad2ML capsite could be detected from pSVA34 (not shown), in agreement with our previous report (17). Cotransfection into Hela cells of pSVA34 together with a recombinant containing the Ad2 left-end EcoRI-PvuII fragment did not result in any detectable transcription from the Ad2MLP (not shown), which demonstrates that the E1A far upstream sequences exert their stimulatory effect only in cis .

An interesting difference emerges from a comparison of the RNA species transcribed from pEIASV (figure 2 and 3) and pEIA-A34 (fig. 4). There is more RNA initiated from the upstream sites in pEIA-A34 than in pEIASV, when compared to RNA initiated from the main capsites.

To identify the E1A left-end region responsible for the activation of the Ad2MLP element, the EcoRI-BalI, EcoRI-SacII and BalI-SacII fragments were deleted from pEIA-A34 (dlEB-A34, dlES-A34 and dlBS-A34, respectively, see figure 4A). It is clear from the results shown in figure 4B that the EcoRI-BalI deletion resulted in an almost complete disappearance of RNA initiated both from the Ad2ML capsite and E1A upstream sites (compare lanes 1 and 2, and lanes 4 and 6). No RNA could be detected when the deletion was further extended to the SacII site (lanes 3 and 7). On the other hand, deletion of the BalI-SacII segment alone, reduced RNA initiated from the Ad2MLP by only 3-fold, and did not affect the amount of RNA initiated from the E1A upstream sites (probe end-point band EP in lane 5).

DISCUSSION

Like the promoters of most genes transcribed by RNA polymerase B (26, 27), the E1A transcription unit contains a TATA box element approximately 30 bp upstream from the main capsite (4), that plays an important role in efficient and accurate initiation of transcription (22, 28). Unlike other transcription units (17, 29-33), no promoter element important for efficient transcription has been found between the TATA box and position -170 (28). We have investigated here the possible role of sequences located even further upstream by transfecting Hela cells with various plasmid recombinants containing the whole E1A transcription unit (pEIASV) or its promoter region alone (pEIA$^-$). Using both recombinants it is clear from our results that sequences located upstream from position -231 (BalI site) are very important for efficient transcription. There is, however, a marked difference in the relative effects of deletions depending on whether the E1A

structural gene is present (pEIASV) or absent (pEIA$^-$). Deletion of the first 270 bp at the Ad2 left-end resulted in a 3-fold decrease of the amount of RNA initiated from the E1A main capsite in the recombinant containing the E1A structural sequence (dlEB) and in a 20-fold decrease in the recombinant lacking this sequence (dlEB$^-$). In contrast further deletion down to position -145 (EcoRI-SacII deletion, dlES and dlES$^-$) resulted in a similar decrease in each case (approximately 5-fold).

Why the EcoRI-BalI fragment is much less important in the presence of the E1A structural sequences is unknown. Further studies are in progress to investigate whether the E1A structural gene contains an enhancer-like element (34) or if the expression of the E1A transcription unit is stimulated by one of its products, as suggested by their known stimulatory effect on the expression of other transcription units (see above). Such a stimulation may account for the 10-fold decrease in the amount of RNA initiated from the E1A promoter when the E1A structural sequences were deleted (pEIA$^-$ vs pEIASV), although a lower stability of RNA transcribed from pEIA$^-$ may also contribute to this decrease (15).

The present study shows that the E1A upstream initiation sites which were first detected by Osborne, Gaynor and Berk (22, 23) early in Ad2 infection are also seen after transfection of E1A containing recombinants into HeLa cells. It is clear from the results obtained with the deletion mutant dlEB (figure 3B) that the promoter elements required for efficient transcription from these upstream sites are contained within the EcoRI-BalI fragment. In this respect it is interesting that deletion of the region which contains two of these upstream sites (see figure 1B, deletion BalI-SacII; mutant dlBS, in figure 3B) does not significantly decrease the total amount of "upstream-initiated" RNA.

The activator effect of the far upstream sequences contained in the EcoRI-BalI fragment of the E1A promoter is reminiscent of the effect of the SV40 72 bp repeat enhancer (17, 35-37). The results obtained with the chimeric recombinant pEIA-A34 and its deletion mutants (figure 4) strongly suggest that these far upstream sequences contain an enhancer-like element, since they can potentiate transcription from a heterologous promoter element located at least 240 bp downstream. It is striking that the EcoRI-BalI deletion has the same drastic effect on initiation from the Ad2MLP in the hybrid recombinant pEIA-A34 as on initiation from the E1A capsite in recombinant pEIA$^-$ that lacks the E1A structural sequences. It is also remarkable that the BalI-SacII deletion in pEIA-A34 (dlBS-A34, figure 4) resulted in an

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