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The E1b promoter of Ad12 in mouse L tk$^-$ cells is activated by adenovirus region E1a

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We have investigated the effect of the E1a region of adenovirus on the expression of the E1b transcriptional unit in mouse L tk$^-$ cells. To that end we have fused the promoter region of the E1b gene of Ad12 to the coding sequence of the thymidine kinase gene of herpes simplex virus type 1. We found that expression of this fusion gene is completely dependent on the presence of the E1a region. Sequences involved in this regulation were mapped between positions −135 and +11 from the E1b cap site. In addition, we found that the largest E1a protein is essential in this regulation process.
Key words: adenovirus-early region 1/E1b-*tk* fusion gene/transfection/E1a mutant

Introduction

Early region 1 (E1) of human adenoviruses has been shown to be responsible for the transforming capacity of the virus or of its DNA (van der Eb *et al.*, 1980). This region consists of two transcriptional units, E1a and E1b. In lytic infection the expression of the E1b region is under control of the E1a region (Berk *et al.*, 1979; Jones and Shenk, 1979) and evidence has been presented that control may occur either at the level of transcription (Nevins, 1981) or at the level of the stability of the E1b RNA (Katze *et al.*, 1981; Persson *et al.*, 1981). We have investigated whether such a control also exists in stably transformed cells and whether the E1b promoter is the target of control by the E1a region. To measure the activity of the E1b promoter we fused this promoter to the protein-coding sequence of the thymidine kinase (*tk*) gene of herpes simplex virus-1 (HSV-1; Figure 1). This fusion gene was transfected into mouse L tk$^-$ cells, both in the presence and the absence of E1a DNA. We found that expression of this gene is completely dependent on the presence of E1a DNA. In addition, we have mapped the sequences involved in the activation of the E1b promoter within 135 bp 5′ from the E1b cap site.

In cells transformed by adenovirus type 12 (Ad12) virions or Ad12 DNA, the Ad12 E1a region specifies two mRNAs with identical 5′ and 3′ termini but differing in the amount of internal sequences removed by RNA splicing (Sawada and Fujinaga, 1980). We observed that a mutation affecting only the larger of these RNAs prevents the activation of the E1b promoter.

Results

To measure E1b promoter activity *in vivo* we have fused the E1b promoter to the coding sequence of the HSV *tk* gene. The construction of this gene is described in Materials and methods and is presented schematically in Figure 1. We have fused a fragment containing the entire protein coding sequence of the *tk* gene but lacking the *tk* transcription initiation site, to a segment containing the E1b promoter of Ad12 with the E1b transcription initiation site (Bos *et al.*, 1981). The promoter fragment contains a 5′-flanking sequence of 520 bp derived from Ad12. This fusion gene, called pE1b-tk/-520, was transfected into mouse L tk$^-$ cells both in the presence and absence of E1a DNA from different Ad serotypes, and cells were selected for the expression of thymidine kinase. Table I shows that colony formation is almost completely dependent on the presence of an E1a region. The E1a region of Ad12 can enhance the frequency of conversion by the E1b-*tk* fusion gene by at least a factor of 10 while the E1a region of Ad5 can enhance it to even higher levels.

We have verified whether indeed the E1b promoter was activated and transcription started at the predicted cap site by mapping the 5′ end of E1b-*tk* RNA by S1 nuclease analysis. The outline of this experiment is presented diagrammatically in Figure 2. A 793-bp *Acc*I-*Bam*HI fragment was isolated from pE1b-tk/-135, a cloned fusion gene with only 135 bp 5′-flanking sequences. This fragment was 5′ end-labeled at the *Acc*I site and hybridized to RNA from cells converted

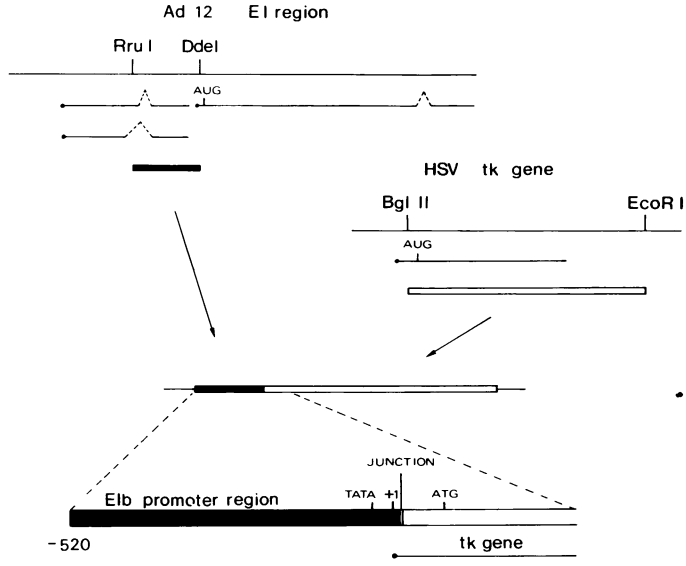

Fig. 1. Organization of pE1b-tk/-520. For details see text.

Table I. Conversion of mouse L tk$^-$ cells by pE1b-tk/-520[a]

DNA	Average colonies/dish[b]
pE1b-tk/-520	0.5
pE1b-tk/-520 + E1a Ad12	23
pE1b-tk/-520 + E1a Ad5	106
pE1b-tk/-520 + HSV *tk*	163

[a]For details see Materials and methods.
[b]Indicated are the average values of nine dishes in three separate experiments.

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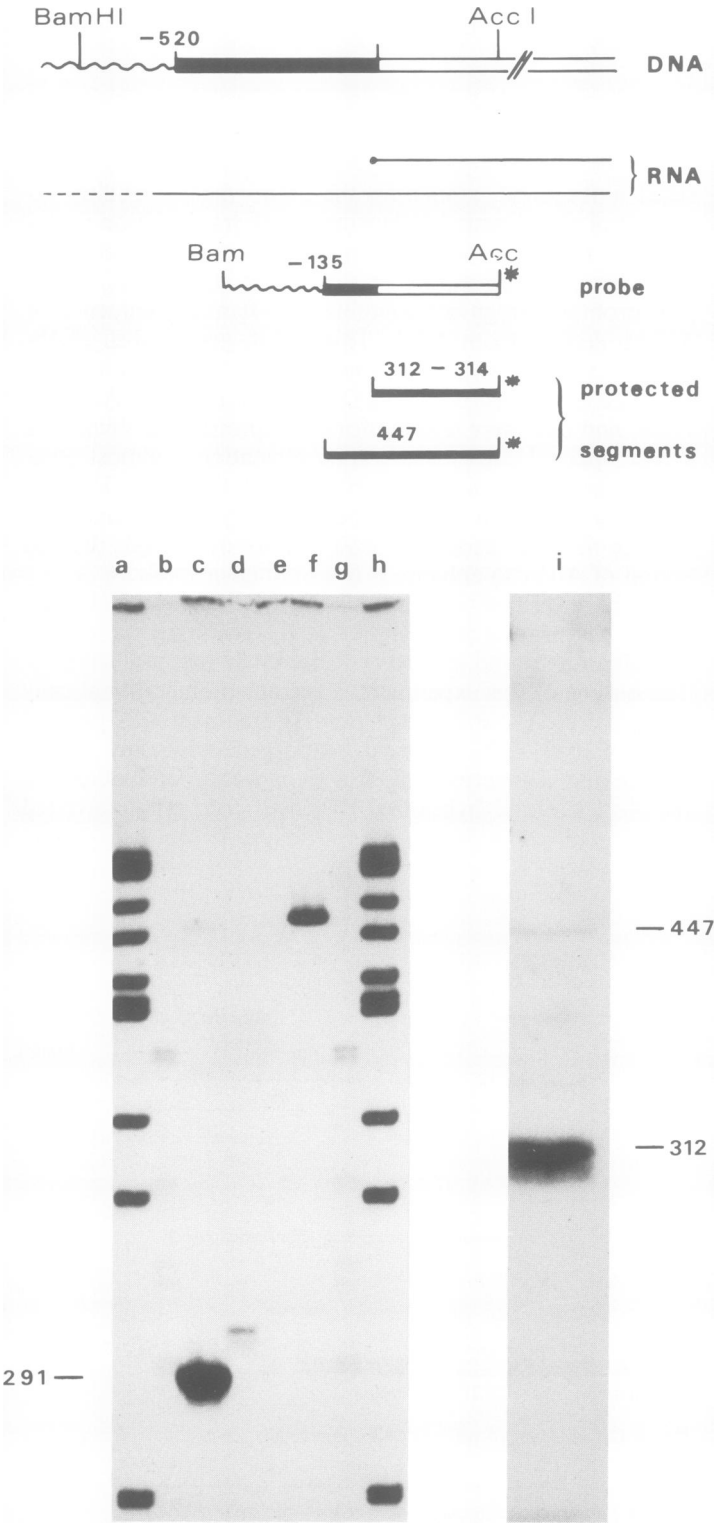

Fig. 2. S1 nuclease mapping. A 5′ end-labeled *Acc*I-*Bam*HI fragment isolated from pE1b-tk/-135 was hybridized to total cytoplasmic RNA of L tk$^-$ cells which were converted to tk$^+$ by pE1b-tk/-520 in the presence of the E1a region of Ad5. The hybrids were treated with S1 nuclease and separated on a 5% polyacrylmide 7 M urea gel. The diagram indicates that possible S1 nuclease resistant products. The autoradiogram shows in **lane a** and **h**: M13 mp8 digested with *Taq*I; **lane b** and **g**: pBR322 digested with *Hin*fI; **lane c**: the 291-bp *Acc*I-*Sau*3A fragment. **Lane d**: S1 nuclease protected segments after hybridization with RNA from cells converted by pE1b-tk/-520 in combination with Ad5 E1a. **Lane 3**: S1 nuclease protected segments after hybridization without RNA. **Lane f**: 5′ end-labeled *Acc*I-*Bam*HI fragment; **lane i**: detail of **lane d**.

with pE1b-tk/-520 in combination with Ad5 E1a DNA. When the messenger of the fusion gene is initiated at the E1b cap site (at position 1525 and 1527 of the Ad12 sequence; Bos *et al.*, 1981) we expect that S1 nuclease treatment will yield an S1 nuclease-resistant segment of 312 – 314 nucleotides. RNAs which are initiated further upstream would yield a protected segment of 447 nucleotides. To obtain an accurate size marker, we have digested the labeled *Acc*I-*Bam*HI fragment with *Sau*3A. This gives a labeled fragment of 291 bp (see Figure 2, lane c). As shown in Figure 2 (lanes d and i) protection of the probe by E1b-tk RNA against S1 nuclease yielded a major product of ~310 nucleotides. This shows that most of the RNA starts at the E1b cap site and thus that the Ad12 E1b promoter is active. The presence of larger S1 nuclease-resistant fragments, however, shows that some of the E1b-tk RNAs start at positions mapping further upstream.

To narrow down the region that is necessary for the control of E1b expression we have constructed a series of derivatives of pE1b-tk/-520 in which increasingly longer segments of the E1b promoter were removed. These plasmids were transfected into mouse L tk$^-$ cells. Table II shows that E1a-controlled expression is still observed when only 135 bp upstream from the E1b promoter (pE1b-tk/-135) were left, whereas deletions up to – 102 abolish E1b promoter activity. This implies that sequences involved in activation of the E1b promoter are located between – 135 and the E1b-*tk* junction at +11.

Finally, we have investigated which of the products encoded by the E1a region of Ad12 is involved in the activation of the E1b promoter. To this end, a mutant plasmid was constructed containing a deletion in one of the two overlapping E1a genes (see Figure 3). This deletion mutant -pR8- has lost 6 bp around the *Rru*I site at position 1003 and affects only the larger E1a messenger (13S) and thus the largest E1a protein. Transfection experiments with pE1b-tk/-135 in com-

Table II. Conversion of mouse L tk$^-$ cells by pE1b-tk plasmids

DNA	Average colonies/dish[b]	
	– E1a Ad5	+ E1a Ad5
pE1b-tk/-520[a]	0.5	106
pE1b-tk/-135[a]	0.5	114
pE1b-tk/-102[a]	1.0	1.5
pE1b-tk/-83[a]	0	0

[a]The numbers refer to the length in base pairs of the 5′-flanking segment of the E1b-*tk* fusion gene, measured from the E1b transcription initiation site.
[b]See legend to Table I.

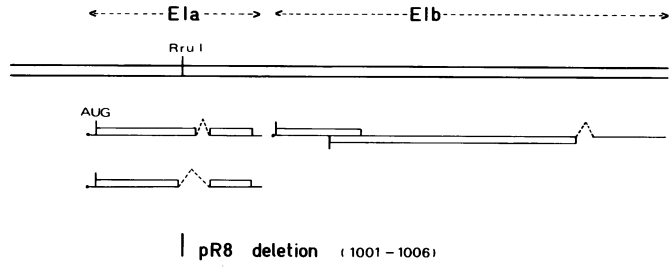

Fig. 3. Schematic representation of the early region 1 of Ad12 and the position of the pR8 deletion.

bination with pR8 DNA (this DNA contains in addition to the mutated region an intact E1b region) shows that the deletion abolishes the capacity of E1a to activate the E1b promoter (Table III). We therefore, conclude that the E1a protein encoded by the large E1a messenger is essential for E1b activation in mouse L tk$^-$ cells.

Discussion

When the E1b promoter of Ad12 is linked to the coding sequence of the *tk* gene of HSV-1 the E1b promoter was completely dependent on the presence of E1a DNA. This shows that in stably converted cells the activity of the E1b promoter is controlled by the E1a region implying that E1a regulates E1b at the level of transcription initiation. These results are in agreement with those obtained for cells lytically infected with Ad5. In the latter case, it was found that the E1a region facilitates expression of the other early regions, including E1b (Berk *et al.*, 1979; Jones and Shenk, 1979), although it was still unclear whether E1a regulates E1b at the level of transcription initiation or at the level of mRNA stabilization (Nevins, 1981; Katze *et al.*, 1981). Our results, however, strongly support the assumption that control occurs at the level of transcription initiation, although our system does not exclude an effect on RNA stability.

The mechanism of activation of the hybrid *tk* gene is not known but it is reasonable to assume that it is identical to that found in lytic infection (Nevins, 1981). This assumption is supported by the finding that both in stably converted cells (this paper) and in lytically infected cells (Berk *et al.*, 1979; Carlock and Jones, 1981; Ricciardi *et al.*, 1981) the gene product encoded by the large E1a messenger (13S) is involved in this regulation. Nevins (1981) has presented evidence that control of expression of region E1b by E1a occurs in an indirect way. Presumably, an E1a gene product inactivates a cellular repressor which prevents the expression of the E1b region. If the action of E1a is indeed mediated by some cellular factor it would suggest that the mechanism by which the E1b promoter is activated represents a general regulatory process of cellular gene expression. This hypothesis is supported by the recent finding of Nevins (1982) that the E1a region can switch on the expression of a 70-kd cellular protein in HeLa cells. Other explanations, however, cannot be excluded.

As shown in Table I, the E1a region of Ad5 has a much higher capacity to activate the E1b promoter than E1a of Ad12. This phenomenon may well correlate with the earlier findings that the frequency of morphological transformation is much higher for Ad5 than for Ad12 (van der Eb *et al.*, 1980), and that this difference resides in the respective E1a regions (Bernards *et al.*, 1982). Since the E1a region alone can transform cells (van der Eb *et al.*, 1980) this correlation suggests that an activation of cellular genes by the E1a region, like the activation of the E1b region, is one of the main causes of cellular transformation.

An implication of our finding that the sequences involved in regulated expression of the E1b genes lie within 135 bp upstream from the 5′ cap site is that only a short region immediately flanking a gene is sufficient for control of expression. In an attempt to identify sequences involved in regulation of expression we have compared the primary structures of the E1b promoter region in three different serotypes, Ad5, Ad7 and Ad12. In this otherwise heterogeneous region we found a rather homologous stretch of 10 bp ~50 bp upstream from the cap site (Van Ormondt, personal communication; Tooze, 1980). The core of this sequence, GGGPyG, is also present at a similar position in all the other adenovirus genes investigated so far (Tooze, 1980) that are under positive control by the E1a region (Figure 4). This sequence might be an essential part of the control region of E1a-regulated genes.

Table III. Conversion of mouse L tk$^-$ cells by pE1b-tk/-135

DNA	Average colonies/dish[b]
pE1b-tk/-135	0.5
pE1b-tk/-135 + pR8	1
pE1b-tk/-135 + pRIC Ad12[a]	31

[a]Wild-type E1a + E1b region.
[b]See legend to Table I.

Ad 12 E1b	−49	T G G G G C G G G C	−40
Ad 7 E1b	−46	T T G G G T G G G G	−37
Ad 5 E1b	−51	T T G G G C G T G G	−42
Ad 2 EII early	−47	A A G G G C G C G A	−38
Ad 2 EIII	−53	C A G G G T G G G T	−44
Ad 2 EIV	−73	C T G G G T G T T T	−64
Ad 5 major late	−52	C C G G G T G T T C	−43
Consensus sequence:		G G G P_YG	

Fig. 4. Comparison of the 5′-flanking sequences of genes regulated by region E1a (Tooze, 1980). The sequences are aligned according to the GGGPyG sequence. The positions of the sequences with respect to the transcription initiation site are indicated.

Materials and methods

Construction of pE1b-tk plasmids

Clone pE1b-tk/-520 was constructed as follows: pAd12 RIC, a clone containing the *Eco*RI C fragment (0–16%) of Ad12 (Bos *et al.*, 1981), was partially digested by *Dde*I. The sticky ends were filled with DNA polymerase I (large fragment) and four dNTPs in order to obtain blunt ends. After cleavage with *Eco*RI a 1534-bp *Eco*RI-*Dde*I fragment (0–1534) was isolated and cloned into *Hind*III- and *Eco*RI-digested pAT153. The *Hind*III site had been filled in to get blunt ends. The isolated clone was digested with *Hind*III (the *Hind*III site had been regenerated at the *Dde*I-*Hind*III junction) and the sticky ends were filled in to obtain blunt ends; subsequently it was further digested with *Bam*HI. The resulting vector (*Hind*III (blunt)-*Bam*HI) was used to clone the 2275-bp *Bgl*II (filled in to yield blunt ends)-*Bam*HI fragment from M_2, a clone that contains the *tk* gene of HSV-1 (Wilkie *et al.*, 1979). From the resulting clone an *Rru*I (position 1003 of Ad12) – *Eco*RI (downstream from the *tk* gene) fragment was isolated and cloned into pAT153. (pAT153 was cleaved with *Cla*I, filled in to obtain a blunt end and subsequently digested with *Eco*RI.) This clone was called pE1b-tk/-520. The site of the E1b-*tk* junction was verified by DNA sequence analysis. The sequence from the former *Dde*I site (Ad12) to the former *Bgl*II site (*tk* gene) was found to be CTTAAGCTGATCT.

Clones with shorter 5′-flanking Ad12 sequences were constructed from pE1b-tk/-520 by cleavage with *Hind*III followed by *Bal*31 digestion. The DNA was subsequently cleaved with *Eco*RI and the fragment containing the

tk gene was isolated and cloned into pAT153 which had been cleaved with *Cla*I (filled in to yield blunt ends) and *Eco*RI. Clones with 5'-flanking Ad12 sequences of 135, 102, and 83 bp, respectively, as measured from the E1b cap site, were isolated.

Transfection

Mouse L tk^- cells were seeded in 5 cm dishes to 5% confluence and grown in Dulbecco's modified Eagle's medium supplemented with 8% newborn calf serum. 24 h later the cells were transfected (van der Eb and Graham, 1980) with 0.5 μg pE1b-*tk* DNA and 12.5 μg high mol. wt. salmon sperm DNA either with or without 1 μg of region E1a DNA. For Ad12 E1a, a cloned *Acc*I H fragment (0–1596; Tooze, 1980) was used and for Ad5 E1a a cloned *Hpa*I E fragment (0–1572; Tooze, 1980). The HSV-tk plasmid was a cloned *Bam*HI fragment from M_2 (Wilkie *et al.*, 1979). 4 h after transfection the medium was changed, and after an additional incubation for 24 h, the cells were exposed continuously to growth medium containing 12 μg/ml hypoxanthine, 1 μg/ml aminopterin and 8 μg/ml thymidine (HAT). Colonies were counted after 15 days of exposure to HAT medium.

S1 nuclease analysis

Total cytoplasmic RNA was hybridized to a 5' end-labeled *Acc*I-*Bam*HI fragment in 20 μl 80% formamide, 40 mM Pipes (pH 6.4), 1 mM EDTA and 0.4 M NaCl at 62°C for 16 h. Hybrids were then incubated for 1 h at 37°C with 300 units of S1 nuclease in 300 μl of 0.25 M NaCl, 30 mM Na acetate, 1 mM $ZnSO_4$ and 150 μg alkali-denatured salmon sperm DNA. Protected DNA fragments were subsequently run on a 5% DNA sequence gel.

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