An enhancer-like element in the adenovirus E2 promoter contains sequences essential for uninduced and E1A-induced transcription

(*trans*-activation/position-independent promoter)

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ABSTRACT Using a chloramphenicol acetyltransferase gene under the control of a series of adenovirus E2 promoter mutants, we have determined the sequence requirements for E1A-induced as well as uninduced expression of the E2 promoter. Fully induced expression requires 79 nucleotides of upstream sequence, and the same sequences are required for uninduced expression. A promoter containing 70 nucleotides of upstream sequence is inducible, although to a lower level than wild type, while a promoter containing 59 nucleotides of upstream sequence is not inducible. The sequences required for induction as well as for uninduced promoter activity map between 21 and 79 nucleotides upstream of the transcriptional initiation site and function in either orientation and at a distance. We suggest that E1A-mediated induction involves the effective increase in a factor utilizing this upstream site and that the factor can functionally interact with the site irrespective of its location relative to the start of transcription.

The transcriptional control of eukaryotic protein-coding genes plays a major role in determining the final level of product (1). Much has been learned recently about the promoter regions of such genes through advances in cloning technology and the ability to reintroduce genes into mammalian cells. Three distinct segments of DNA have been identified that participate in the initiation of transcription (2–4). The “TATA” element, a highly conserved sequence found 25–30 nucleotides upstream of the start site, is essential for correctly positioning the initiation of transcription. Further upstream, sequences have been identified that are required for efficient transcription of the gene. In contrast to the TATA element, these upstream sequences can often function irrespective of orientation relative to transcription and in some cases can be displaced from the transcriptional initiation site and retain function (5–8). Finally, in certain genes there are additional sequence elements referred to as enhancers that are also required for normal, efficient transcription. In contrast to the other elements of the promoter, the enhancer may be found internal to the gene (9–11). It can also function irrespective of its position or orientation relative to the transcription initiation site.

During a lytic viral infection, transcription of the adenovirus E2 gene is stimulated by a protein product of the viral E1A gene; there is, in fact, not an absolute requirement for E1A but, rather, E1A appears to increase transcription from a low basal level (12–14). We and others have previously reported the sequence requirements for the fully induced expression of the adenovirus E2 gene (15, 16). We found that, in the presence of E1A, 79 nucleotides of upstream sequence were sufficient for wild-type expression of the E2 gene. We also found that we could restore activity to a mutant promoter retaining only 28 nucleotides of upstream se-

quence by inserting a fragment that contained the sequences from –21 to –262 of the wild-type gene. Furthermore, these sequences could function in either orientation at the –28 position or could be placed at a distance of >4 kilobases (kb) from that site. Thus, the region of DNA from –21 to –79 has all the properties of an enhancer element when assayed in the presence of E1A induction.

Although it was clear that sequences between –21 and –79 were essential for E2 expression, the role of these sequences in relation to E1A induction was not defined. For example, it was possible that the upstream enhancer-like element was the basic promoter element of the transcription unit, while a regulatory site required for E1A induction was internal to the gene. Alternatively, sequences required for both basal and induced activity could be identical and reside within the upstream element. To explore such questions, we have taken two approaches. First of all, we have made use of a more sensitive assay to accurately measure uninduced expression in order to determine the basal promoter activity of the upstream sequences. Second, we have inserted the upstream enhancer-like element into a heterologous promoter to assay for E1A induction in the absence of any internal E2 sequences. The results indicate that the E2 promoter region and the sequences required for E1A induction are essentially inseparable and have the qualitative properties of an enhancer element.

MATERIALS AND METHODS

Cells. HeLa cells and 293 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Transfections. Transfections and CAT assays were carried out as described (17).

Hypoxanthine/Aminopterin/Thymidine (HAT) Selection. Selection of thymidine kinase positive (tk⁺) colonies in HAT medium was carried out as described (18).

Plasmids. The pEC series of E2-CAT plasmids was constructed as follows. A *Sau*3A fragment that spans the *Eco*RI site of either pE2 or the pE2 deletion mutants described (15) was cloned into the unique *Bgl* II site of pCAT3M (see Fig. 1). pCAT3M contains the chloramphenicol acetyltransferase (CAT) gene without any transcriptional initiation signals or upstream elements (gift of L. Laimins). Recombinant plasmids were screened to ensure the proper orientation of the *Sau*3A fragment by restriction mapping of mini-lysates (19).

The pHf series of plasmids were constructed as follows. The *Bam*HI insert of plasmid pMHf (15), which contains the E2 upstream *Hinf*I fragment from –262 to –21 with *Bam*HI linkers, was cloned into either ptk/5'–32 at the *Bam*HI site (to yield pHf_{tk} and pHf'_{tk}) or pA₁₀CAT4N at the *Bgl* II site (to yield pHf_{CAT} and pHf'_{CAT}). ptk/5'–32 contains a *Her*-

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Abbreviations: CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; SV40, simian virus 40; kb, kilobase(s); bp, base pair(s).

pes simplex virus *tk* gene in which all the upstream sequences except for the TATA box have been removed (20). pA₁₀CAT4N contains the CAT gene under the control of a simian virus 40 (SV40) promoter region in which all of the SV40 upstream sequences, including the 21- and 72-base pair (bp) repeats, have been removed, leaving only the TATA box (gift of N. Rosenthal). Recombinants were screened for the orientation of the insert by restriction digestion of DNA from mini-lysates.

pE1A was constructed from pGC212 (17) by deleting the E1B sequences from the *Sst* I site at 4.9 map units to the *Hind*III site at 7.8 map units.

RESULTS

Assay of E2 Promoter Activity Using a CAT Construction.

In previous experiments, we used an intact E2 gene, and the assays consisted of measuring production of the 72-kDa E2 protein in immunoblots (15, 17). Unfortunately, the sensitivity was not sufficient to easily detect uninduced levels of expression after transfection into HeLa cells. To alleviate these problems, we cloned the E2 promoter region from the wild-type gene and the various deletion mutants upstream of the protein coding sequences of the bacterial CAT gene, as shown in Fig. 1. For the wild-type promoter, such a construction contains 285 nucleotides of E2 upstream sequence and 40 nucleotides downstream from the cap site. The plasmids derived from the various mutant promoters contain the same downstream sequence but various amounts of upstream sequence. Furthermore, transfection of these plasmids into HeLa cells now allows an accurate measurement of promoter function in the absence of induction. We have shown that these plasmids are indistinguishable from the original set of E2 plasmids in that the level of expression in transfections is linear over the same range of input DNA, and the wild-type plasmid is inducible in a cotransfection by both the E1A gene and the pseudorabies virus immediate early gene (data not shown). Therefore, such E2-CAT constructions allow sensitive measurements to be made of uninduced expression and retain E1A inducibility.

Role of E2 Upstream Sequences in Uninduced and Induced Expression. Our previous experiments demonstrated that 79 nucleotides of upstream sequence were sufficient for fully induced E2 expression, while a promoter containing only 70 nucleotides was severely impaired (15). Such a result could

FIG. 1. Construction of E2-CAT genes. A *Sau*3A fragment spanning the E2 promoter was cloned into the *Bgl* II site of pCAT3M. The resulting plasmid containing the wild-type E2 promoter was termed pEC. Constructions derived from deleted promoters were named accordingly (i.e., pEC-97 derives from the pE2 promoter deleted to -97) (15).

be due to two possibilities. First, a regulatory site between -79 and -70 may have been affected such that the gene was no longer inducible. In such a case, the basal uninduced activity would remain constant in the two mutants. Alternatively, such a site might represent the essential promoter element of this gene so that uninduced expression would be affected to the same level as induced expression. To investigate this issue, we have assayed the various mutant promoters in 293 cells (induced) or in HeLa cells (uninduced). Forty-eight hours after transfection, protein extracts were prepared and CAT assays were performed (Fig. 2). As shown in Fig. 2A, transfection and assay of the mutant E2-CAT plasmids in 293 cells revealed essentially the same pattern of expression as we previously found with the E2 plasmid mutants (15). That is, induced expression required 79 nucleotides of upstream sequence, although we often found that the -79 promoter was reduced compared to the wild-type promoter (≈ 2 -fold). The activity of the -70 promoter was $\approx 5\%$ that of the wild-type promoter. Thus, the border of sequences critical for induced activity fall in the -70 to -79 region. Activity of the various promoters was then assayed in the absence of E1A induction by transfection into HeLa cells (Fig. 2B). The results were very similar to those from 293 cells. Expression was nearly constant with the wild-type promoter, the -97 promoter, and the -79 promoter, although once again, the -79 promoter was reduced somewhat compared to wild type by ≈ 2 -fold. In contrast, activity of the -70 promoter was $\approx 5\%$ that of wild type. Therefore, the same sequences appear to be required for both induced and uninduced expression of the E2 promoter.

Similar transfections into HeLa cells were set up in the presence and absence of the plasmid pE1A, which contains

FIG. 2. Uninduced and induced activity of E2 promoter deletion mutants. Each of the pEC plasmids (2 μ g) was transfected into either 293 cells (A) or HeLa cells (B). Extracts were prepared 48 hr later and assayed for CAT activity. Ac-CM, acetylated chloramphenicol; CM, chloramphenicol.

FIG. 3. Induction of E2 promoter deletion mutants by E1A. Each of the pEC plasmids was transfected into HeLa cells in the presence (+) or absence (-) of 16 μ g of pE1A. Extracts were prepared and assayed for CAT activity 48 hr later. Ac-CM, acetylated chloramphenicol; CM, chloramphenicol.

the adenovirus 2 E1A gene. Once again, CAT activity was assayed 48 hr after transfection. In the presence of E1A, the wild-type, -97, and -79 mutants were induced to nearly equal levels (Fig. 3), consistent with the assays in 293 cells. The -70 mutant was also inducible but to a lower level than the wild-type gene. However, since the basal level was also reduced, the degree of induction was nearly the same as for the other promoters. In contrast, the -59 promoter and the -17 promoter were clearly not inducible by E1A. These results indicate that deletion from -79 to -70 severely impairs the function of the promoter but still allows induction, whereas deletion to -59 totally inactivates the promoter, including its ability to respond to E1A stimulation.

Sequences Required for Uninduced Expression Can Function Independent of Position or Orientation. We previously demonstrated that a DNA fragment from -262 to -21 of the E2 promoter could restore activity to an E2 gene with a promoter containing only 28 nucleotides of upstream sequence (-28 promoter) (15). Furthermore, this fragment could do so in an orientation- and position-independent manner. Since those assays were under induced conditions, we could not determine the relative contributions of this region to basal activity and induction. We have now determined whether the -21 to -262 fragment could supply promoter activity to a gene lacking an upstream element and in the absence of E1A induction. For such assays, we used a mutant herpesvi-

rus *tk* gene that possesses only 32 nucleotides of upstream sequence (20). The E2 -21 to -262 fragment was inserted into the *tk* mutant at the -32 position in both orientations (Fig. 4). Each of these constructions was then assayed for activity by transfection into *tk*⁻ L cells and selection of *tk*⁺ colonies in HAT medium. As shown in Table 1, the -32 *tk* plasmid possessed very little activity, while each of the constructions containing the E2 -21 to -262 fragment produced a substantial increase in the number of *tk*⁺ colonies, although neither E2 construction was as active as the wild-type *tk* gene. Since both of these constructions were functional, it was clear that the E2 element could function independently of its orientation in the absence of E1A induction. Furthermore, since the construction that inverts the E2 fragment displaces the critical sequences of -21 to -79 by $\approx$ 200 nucleotides, these sequences can also function at a distance.

Sequences Required for E1A Induction. To determine whether the E2 upstream enhancer-like element possesses sequences required for E1A induction, as suggested from the analysis of 5' deletion mutants, we constructed another series of recombinant plasmids as shown in Fig. 5A. We cloned the E2 fragment upstream of a *CAT* gene under the control of an early SV40 promoter containing only the TATA element. These plasmids were then used to determine whether the -21 to -262 fragment could confer E1A inducibility to the *CAT* gene. Each of these plasmids, as well as the wild-type E2-CAT gene (pEC), was transfected into HeLa cells in the presence or absence of pE1A, and CAT activity was assayed 48 hr later. The results are depicted in Fig. 5B and Table 2. The starting plasmid, pA₁₀CAT4N, gave low uninduced expression and no induction by E1A. pHfCAT, which contains the E2 upstream element in the proper orientation, was

FIG. 4. E2-*tk* constructions. The E2 upstream *Hinf*I fragment from -262 to -21 was cloned into *ptk/5'-32*. Recombinants were isolated containing the E2 fragment in the normal orientation (pHftk) or in the opposite orientation (pHf'tk).

Table 1. Role of E2 upstream enhancer element in promoter function

Experiment	Plasmid	Colonies, no.
1	<i>ptk/5'-32</i>	3
	pHftk	257
	pHf'tk	62
2	<i>ptk/5'-32</i>	13
	pHftk	139
	pHf'tk	120
3	<i>ptk/5'-32</i>	<1
	pHftk	62
	pHf'tk	6
	<i>ptk</i> (wild type)	334

Each of the plasmids was transfected into Ltk⁻ cells as described (17), and *tk*⁺ colonies were selected for as described. Experiments 1, 2, and 3 received 200 ng, 1 μ g, and 20 ng, respectively, of plasmid DNA per 100-mm dish.

FIG. 5. Role of the E2 upstream region in E1A induction. (A) Schematic diagram of the construction of HfCAT plasmids. Recombinants were isolated containing the E2 fragment in the normal (pHfCAT) or opposite (pHf'CAT) orientation. (B) pA₁₀CAT4N, pHfCAT, pHf'CAT, or pEC (2 μ g each) were transfected into HeLa cells in the absence (–) or presence (+) of 16 μ g of pE1A. Extracts were prepared 48 hr later and assayed for CAT activity.

about as active as the starting plasmid and was only marginally inducible by E1A (no more than 2-fold). The plasmid pHf'CAT, which contains the E2 upstream element in the opposite orientation, was also about as active as the starting plasmid but was significantly induced by E1A. The induction with this construction averaged 10-fold. Although it is not clear why the pHfCAT plasmid was not induced by E1A to any significant levels, it does appear that these E2 upstream sequences can confer E1A inducibility. Therefore, we conclude that the E2 upstream element contains sequences that allow induction by E1A and that these sequences can function at a distance and in the opposite orientation.

DISCUSSION

We have defined the sequence requirements for the transcription of the adenovirus early region 2 (E2) gene both in the presence and in the absence of E1A. Previously, we examined the expression of upstream deletion mutants in 293 cells, adenovirus-transformed cells that express the E1A gene constitutively. We demonstrated that 79 nucleotides upstream from the major E2 transcriptional start site were sufficient for wild-type levels of expression in these cells (15). In the absence of E1A, we now find that wild-type lev-

Table 2. Role of E2 upstream element in E1A induction

Plasmid	Exp. 1		Exp. 2		Exp. 3	
	CAT	Ind	CAT	Ind	CAT	Ind
pA ₁₀ CAT4N	0.04		0.19		0.05	
With E1A	0.05	1.3	0.18	0.9	0.08	1.6
pHfCAT	0.05		0.17		0.07	
With E1A	0.13	2.6	0.28	1.6	0.09	1.3
pHf'CAT	0.03		0.17		0.05	
With E1A	0.50	16.7	0.75	4.4	0.47	9.4
pEC	ND		0.22		0.13	
With E1A	ND		5.43	24.7	3.12	24.0

CAT activity is expressed as the percentage conversion of chloramphenicol to acetylated chloramphenicol. Induction (Ind) is expressed as the ratio of CAT activity in the presence of E1A to that in the absence of E1A. ND, not determined.

els of uninduced expression are also obtained from plasmids that retain only 79 nucleotides of upstream sequence. There is a slight drop in expression of the –79 mutant in both HeLa and 293 cells, but this decrease is by a factor of <2. Mutants containing only 70 nucleotides or less of upstream sequence, however, function at levels that are only 5% of wild type. In the presence of E1A, either through a cotransfection with a plasmid containing the E1A gene or by transfection into 293 cells, we find that although 79 nucleotides of upstream sequence are still required for wild-type fully induced expression, the mutant that retains 70 nucleotides is still inducible. Mutants that have 59 or fewer upstream nucleotides are not inducible. Therefore, the 5' border of sequences that are important for transcription of the E2 gene fall somewhere between –79 and –70 with respect to the transcriptional start site, both for induced as well as uninduced expression. These results clearly indicate that the critical sequence between 70 and 79 nucleotides upstream is not simply required for induction. Our results are very similar in this regard to previous work of Elkaim *et al.* (16), who showed that induced and uninduced expression required 93 nucleotides of upstream sequence.

These results suggest that the same sequences are required for induced and uninduced transcription, and therefore the same factor might be involved in induced and uninduced transcription of this gene. If this were the case, we would suggest that the function of E1A is to increase the effective concentration of such a factor. Thus, as long as sequences remain that can interact with the factor, the gene would still be inducible by E1A. An alteration in the binding site might reduce the efficiency (e.g., the –70 promoter), but only the total removal of the site (e.g., the –59 promoter) would result in a loss of induction. In this regard, the use of small deletions or point mutations to define sequences important for inducibility must be viewed with caution, because partial removal or alteration of such a site still leaves the promoter inducible. Such a result could not be taken by itself to suggest a separate site for induction or that induction is sequence independent.

An alternative possibility is that there are two sites within the –21 to –79 region that are required for fully induced expression and interact with separate factors. The distal site, extending to near the –79 position, would be required for basal promoter activity. A distinct site involved in induction by E1A would be proximal to the transcriptional start site. The resolution of these two possibilities will require the isolation of the protein factor(s) necessary for uninduced and induced expression.

Finally, the sequences involved in E1A induction as well as in uninduced E2 promoter activity function independently of position or orientation. Perhaps surprising is the finding that the element functions better for E1A induction in the

opposite orientation than in its proper orientation, at least in this particular set of constructions. The reason for this is not clear, but we suspect that it might be due to the juxtaposition of the normal SV40 TATA element with an E2 TATA-like element in the construction containing the E2 element in the proper orientation. In previous experiments, we have found that such an arrangement allows initiation events directed by the E2 element (15). Thus, RNA may be produced from this particular construction with an extended 5' end, potentially impairing production of the CAT enzyme.

Other upstream promoter elements such as the 21-bp repeat of the early SV40 promoter (6), the heat shock responsive element of the *Drosophila* hsp70 promoter (5), and the upstream element of the herpes tk promoter (7, 8) have been shown to function with some flexibility with regard to their position and orientation. Transcription factors other than the TATA-specific factor may interact in a functional manner with these DNA sequences irrespective, to various extents, of the location of such sequences relative to the transcriptional start site. Left unclear at this stage is the functional relationship between an enhancer element and the immediate upstream element in a complex promoter such as the early SV40 promoter, since both of these elements appear to interact with protein factors to facilitate the transcription of the gene (21–24).

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