Adenovirus EIIA early promoter: Transcriptional control elements and induction by the viral pre-early EIA gene, which appears to be sequence independent

(adenovirus 5 EIIA early promoter/linker-scanning mutants/chloramphenicol acetyltransferase vector/transient expression assay/trans-activation)

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ABSTRACT A molecular dissection of the adenovirus EIIA early (E) promoter was undertaken to study the sequence elements required for transcription and to examine the nucleotide sequences, if any, specific for its trans-activation by the viral pre-early EIA gene product. A chimeric gene in which the EIIA-E promoter region fused to the coding sequences of the bacterial chloramphenicol acetyltransferase (CAT) gene was used in transient assays to identify the transcriptional control regions. Deletion mapping studies revealed that the upstream DNA sequences up to -86 were sufficient for the optimal basal level transcription in HeLa cells and also for the EIA-induced transcription. A series of linker-scanning (LS) mutants were constructed to precisely identify the nucleotide sequences that control transcription. Analysis of these LS mutants allowed us to identify two regions of the promoter that are critical for the EIIA-E transcription. These regions are located between -29 and -21 (region I) and between -82 and -66 (region II). Mutations in region I affected initiation and appeared functionally similar to the "TATA" sequence of the common studied promoters. To examine whether or not the EIIA-E promoter contained DNA sequences specific for the trans-activation by the EIA, the LS mutants were analyzed in a cotransfection assay containing a plasmid carrying the EIA gene. CAT activity of all of the LS mutants was induced by the EIA gene in this assay, suggesting that the induction of transcription of the EIIA-E promoter by the EIA gene is not sequence-specific.

By using a variety of assays, the essential nucleotide sequences and functional domains of a number of cellular and viral RNA polymerase II promoters have been identified. The majority of these promoters contain two essential control elements. The first such element, the "TATA" box located between 20 and 30 base pairs (bp) upstream from the initiation site, positions the initiation of transcription (1, 2). Another type of element that is usually found between 50 and 150 bp from the initiation site is required for efficient transcription (3–12). Several promoters also carry a third type of control signal termed an enhancer, which is capable of functioning independently of position and orientation (13–19).

Adenovirus (Ad) early transcription region II (EIIA) is an RNA polymerase II transcription unit that exhibits relatively complex organization. At early stages of infection, three messages, one coding for a 51,72,000 DNA binding protein (EIIA) and two coding for two polypeptides involved in DNA replication (EIIB), are transcribed from a single promoter located at 76.0 map units (m.u.), designated EIIA early (E) promoter (20, 21). At intermediate stages after infection, the EIIA region is transcribed from a second new promoter (21). Both of these promoters are somewhat unusual in that they lack TATA box elements (21). At present it is not clear whether these promoters lacking the TATA box utilize in its place an analogous sequence element or whether they belong to a separate class of polymerase II promoters that are differently regulated. The EIIA-E promoter that functions early in infection is also subject to positive regulation. A 289 amino acid phosphoprotein encoded by the pre-early EIA region is required for the activation of the EIIA-E promoter and other early viral promoters following the Ad infection of permissive human cells (22–24).

A detailed mutational analysis of the EIIA-E promoter was undertaken in this investigation to identify the DNA sequences required for transcription and to determine whether or not this promoter contains nucleotide sequences or domains that are responsible for its trans-activation by the viral pre-early EIA gene product. We have constructed a series of linker-scanning (LS) mutants of the EIIA-E promoter after fusing the promoter with the bacterial chloramphenicol acetyltransferase marker gene (CAT; ref. 25). CAT activity of the LS mutants was measured in HeLa cells with and without cotransfection with a plasmid containing the EIA gene. These studies show that the EIIA-E promoter contains two transcriptional control regions upstream from the cap site that are essential for its constitutive expression. Nucleotide sequences specific for the trans-activation by the EIA gene product could not be detected for all of the LS mutants were found to be induced efficiently by the EIA gene.

MATERIALS AND METHODS

Cells and Plasmids. HeLa and 293 cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. pEIIA-E-CAT is a recombinant plasmid in which a 346-bp Ad DNA fragment containing promoter sequences and the sequences corresponding to the first leader segment of the EIIA-E message was fused to the coding sequences of the CAT (25) at the +62 position (relative to the cap site of the EIIA-E gene; see legend to Fig. 1 for the construction protocol). Plasmid pCD2 was a gift from F. L. Graham (Hamilton University) and contains the 0- to 5.0-m.u. fragment from the left end of Ad5 DNA.

Construction of LS Mutants. By using nuclease BAL-31 (26), two libraries of deletion mutants of pEIIA-E-CAT starting from the unique Bgl II and Xho I sites with deletions extending in opposing direction were constructed. Each deletion mutation terminated with a BamHI linker sequence. The end points of the deletions were determined by DNA sequence analysis (27). Matching 5'- and 3'-end deletion mutants were chosen to construct 15 LS mutants.

Abbreviations: CAT, chloramphenicol acetyltransferase; LS, linker scanning; bp, base pair(s); m.u., map unit(s); Ad, adenovirus; E, early; wt, wild type.

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2230
RESULTS

Assay of Promoter Activity Using a CAT Vector. We fused a 346-bp DNA fragment containing promoter sequences and the sequences coding for the first leader segment of the Ad5 EIIA-E specific message to the CAT coding sequences obtained from a derivative of pSV2-CAT, a recombinant vector that expresses CAT in eukaryotic cells (25). In the resulting plasmid, pEIIA-E-CAT [wild type (wt) plasmid], the coding region for CAT is placed under the transcriptional control of the EIIA-E promoter. Organization of the EIIA transcription unit on the Ad chromosome and the structure of the chimeric gene described here are shown in Fig. 1 A and B, respectively. CAT activity has been shown to correlate well with the level of CAT-specific mRNA in cells transfected with a variety of chimeric genes containing this marker gene (25, 28, 29). CAT assay therefore provides a quick, sensitive, and reliable assay for quantitating the expression of the EIIA-E promoter in the studies described here. HeLa cells were transfected with this chimeric gene and, after 48 hr, the conversion of [3H]chloramphenicol to its mono- and diacetylated derivatives by the cell extracts was assayed as described by Gorman et al. (25).

We have consistently observed a significant basal level of CAT activity expressed by pEIIA-E-CAT in HeLa cells in the absence of the EIIA gene product. This EIA-independent basal level of transcriptional activity was used to determine the minimal DNA sequences required for transcription and to identify the transcriptional control regions of the EIIA-E promoter. Fig. 2 and Table 1 show CAT activity of the deletion mutants extending toward the cap site from the 5' end. A deletion mutant that retained 86 bp from the cap site showed a normal level of CAT activity. Deletion mutations extending further toward the cap site showed a drastic reduction of activity. Therefore, 86 bp upstream from the cap site are sufficient for the optimal basal level transcription of the EIIA-E promoter in this assay.

To determine if the 86 bp upstream from the cap site are also sufficient for the EIA-induced transcription, CAT activity of the deletion mutants was assayed in HeLa cells after cotransfecting with a plasmid containing EIA-coding sequences (pCD2). CAT activity of the wt plasmid was induced up to about 8-fold by the EIA gene product (Fig. 2 and Table 1). The deletion mutant containing 86 bp upstream from the cap site was also induced about 5-fold. These results suggest that 86 bp from the cap site are sufficient for both constitutive and induced expression of the EIIA-E promoter. Interestingly, CAT activity of the deletion mutants lacking sequences upstream from the −40 position was also induced about 2- to 3-fold by the EIA gene product.

Identification of Two Transcriptional Control Regions Upstream from the Cap Site. To identify the control signals for trans-activation by the EIA gene product, a detailed mutational analysis of the entire 86 bp of the promoter was essential. We chose the LS mutagenesis procedure described by McKnight and Kingsbury (6) to mutate the EIIA-E promoter. This is the procedure of choice because the method permits the introduction of a clustered set of point mutations in the regions of interest without altering the spacing of the important control signals.

A set of 15 LS mutants that systematically mutate the DNA sequence of the promoter region were mapped; the nucleotide sequences of the wt promoter and the 15 LS mutants are shown in Fig. 3. Of the 15 LS mutants, only 5 have single base additions but none has deletions. HeLa cells were transfected with the LS mutants and, after 48 hr, CAT activity in each case was measured as described (ref. 25; autoradiogram not shown). The relative activities of the LS mutants compared to that of the wt plasmid are given in Table 2.

LS +9/+3 shows a moderate loss of activity. This mutant has 10 of 12 nucleotides substituted around the cap site. We have determined the transcription start sites of this mutant using a nuclear S1 mapping procedure described by Weaver and Weissman (ref. 32; see below). Fig. 4 shows that LS
Table 1. CAT expression of the upstream deletion mutants of the pEIIA-E-CAT and their induction by the EIA gene product

<table>
<thead>
<tr>
<th>Mutant</th>
<th>CAT activity without EIA as percentage of wt</th>
<th>Fold increase in expression by EIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>100.0</td>
<td>7.7</td>
</tr>
<tr>
<td>-100</td>
<td>96.0</td>
<td>3.9</td>
</tr>
<tr>
<td>-86</td>
<td>100.0</td>
<td>4.9</td>
</tr>
<tr>
<td>-83</td>
<td>30.3</td>
<td>5.4</td>
</tr>
<tr>
<td>-73</td>
<td>14.1</td>
<td>4.9</td>
</tr>
<tr>
<td>-69</td>
<td>10.0</td>
<td>3.2</td>
</tr>
<tr>
<td>-64</td>
<td>6.1</td>
<td>2.4</td>
</tr>
<tr>
<td>-51</td>
<td>3.9</td>
<td>2.3</td>
</tr>
<tr>
<td>-48</td>
<td>4.4</td>
<td>1.8</td>
</tr>
<tr>
<td>-40</td>
<td>4.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Values presented here are the average of two experiments. Transfection of monolayer HeLa cells with deletion mutants and with or without EIA plasmids was as described in the legend to Fig. 2. Values for deletion mutant -81 are not shown.

+9/3 protected identical-size DNA fragments as that of wt in the nuclease S1 mapping assay with the exception of a band corresponding to initiation at +1. Therefore, mutations around the EII-A Cap site have minimal effect on transcription.

LS mutants -4/-14 and -10/-21 showed normal or near-normal levels of CAT activity, suggesting that these sequences do not control transcription. A reduction in activity by a factor of 6-7 was obtained for LS mutants -15/-26 and -19/-29 followed by a moderate loss of activity (by a factor of 2) for LS -25/-34 and LS -31/-41. Activity was restored to normal level for LS -35/-46. Clearly, there is a transcriptional control region between -35 and -21 in the EIIA-E promoter. The core sequences of this region (region I) lie between -29 and -21 with a sequence 5'-C-T-T-A-A-G-A-G-T 3'. Interestingly, this control element is approximately the same distance from the start site as the TATA sequence of the major promoter of eukaryotic RNA polymerase II promoters (1). Furthermore, the sequence T-T-A-A of this region has some resemblance to the TATA box.

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Table 2. Relative efficiency of CAT expression of the LS mutants and the induction of CAT activity of the LS mutants by the EIA gene product

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Relative expression* (mutant/wt)</th>
<th>Fold increase in expression by EIA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1.0</td>
<td>7.0</td>
</tr>
<tr>
<td>LS +9/-3</td>
<td>0.55†</td>
<td>5.2</td>
</tr>
<tr>
<td>LS -4/-14</td>
<td>0.99</td>
<td>5.5</td>
</tr>
<tr>
<td>LS -10/-21</td>
<td>1.27</td>
<td>4.1</td>
</tr>
<tr>
<td>LS -15/-26</td>
<td>0.18‡</td>
<td>8.5</td>
</tr>
<tr>
<td>LS -19/-29</td>
<td>0.14‡</td>
<td>10.8</td>
</tr>
<tr>
<td>LS -25/-34</td>
<td>0.58‡</td>
<td>6.1</td>
</tr>
<tr>
<td>LS -31/-41</td>
<td>0.43‡</td>
<td>4.8†</td>
</tr>
<tr>
<td>LS -35/-46</td>
<td>1.09†</td>
<td>3.8</td>
</tr>
<tr>
<td>LS -40/-50</td>
<td>0.82</td>
<td>5.7</td>
</tr>
<tr>
<td>LS -49/-59</td>
<td>5.1†</td>
<td>4.0†</td>
</tr>
<tr>
<td>LS -55/-66</td>
<td>1.35</td>
<td>3.8§</td>
</tr>
<tr>
<td>LS -63/-73</td>
<td>0.11†</td>
<td>3.7§</td>
</tr>
<tr>
<td>LS -65/-75</td>
<td>0.08†</td>
<td>5.1</td>
</tr>
<tr>
<td>LS -74/-85</td>
<td>0.39†</td>
<td>6.6§</td>
</tr>
<tr>
<td>LS -82/-92</td>
<td>0.73†</td>
<td>3.9</td>
</tr>
</tbody>
</table>

CAT activity of the LS mutants was determined as described in the legend to Fig. 3.

*Values presented here are the average of seven experiments, except where indicated.
†LS mutants were cotransfected with pC2. Values presented here are the average of six experiments, except where indicated.
‡Average of six experiments.
§Average of four experiments.

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Fig. 3. Nucleotide sequences of the LS mutants. The single-stranded DNA sequence on the top line represents the nucleotide sequence of the sense strand of the Ad5 wt EIA-E gene. The numbering of the LS mutants shown here represents the nucleotides deleted from the parental 5’ and 3’ deletion mutants that are combined for their construction. Nucleotides mutated are shown in black background. In those mutants that contain single base insertions, the last G of the synthetic linker is positioned above the sequence. Each LS mutant was sequenced in both directions after end labeling at the BamHI site. To ensure that the LS mutants contained a single BamHI linker, end-labeled DNA fragments encompassing the promoter region (Bgl II to Nar I, 68 to -96, respectively) were analyzed on a DNA sequence analysis gel with appropriate standards. For construction of these LS mutants, both 10- and 12-mer BamHI linkers were used. +1 represents the major initiation site identified by earlier workers (21, 31). A nucleotide shown in parentheses at -5 above the wt sequence indicates the difference found in the corresponding Ad2 DNA sequence.
Fig. 4. Nuclease S1 mapping of the 5' ends of RNAs synthesized by the LS mutants. 293 cells (100-mm dishes) were transwcted with 15 μg of the LS mutants as described in the legend to Fig. 2. Ten micrograms of poly(A)⁺ RNA isolated from these cells was annealed with the probe (labeled at Bgl II site; 4-5 × 10⁶ cpm) at 32°C for 16 hr. The hybrids were treated with nuclease S1 [70,000 units/ml (Miles) for 40 min at 15°C], and the protected DNA fragments were analyzed on 10% DNA sequencing gels. (A and B) Data obtained from two separate experiments. Homologous probe was used in the case of LS +9/−3 in A and LS −15/−26 and LS −19/−29 in B. wt probe was used for the remaining samples. Arrowheads at positions 68 and 96 corresponding to markers represent major and minor initiations, respectively (21, 31).

Nucleotide sequences from −66 to −35 do not appear to control transcription because LS mutants −35/−46, −40/−50, −49/−59, and −55/−66 show normal CAT activity (Table 2). For reasons that we cannot explain at present, LS −49/−59 consistently showed much higher activity than the rest of the mutants. In contrast, mutations of nucleotides between −82 and −66 affect transcription. CAT activities of LS mutants −63/−73 and −64/−75 were reduced by factors of 9 and 12, respectively. Transcription is restored to a significant level for LS mutant −74/−85. LS −82/−92 showed only a moderate loss of activity. Therefore, nucleotide sequences from −82 to −66 define a second transcriptional control region of the EIA-E promoter. The DNA sequence for this region is 5' T-G-G-A-G-A-T-G-A-C-G-T-A-G-T-T-T 3'.

Mapping of 5' Ends of mRNAs Encoded by the LS Mutants. To examine whether the function of the control region I is to properly localize the initiation of transcription, we have performed the 5'-end analysis of the RNAs synthesized by these mutants using the nuclease S1 mapping procedure (32). Poly(A)⁺ RNAs synthesized by the LS mutants in 293 cells were probed with an end-labeled DNA fragment (68 to −96, end labeled at Bgl II site). A 68-nucleotide-long band corresponding to initiation at +1 and two or three additional bands were protected from nuclease S1 for RNAs synthesized by the wt plasmid (Fig. 4A). This is consistent with an earlier observation that the major initiation is at +1 in virus-infected cells (21). Additional bands detected in our gels are identical to those observed by Mathis et al. (31) and likely to correspond to authentic start sites.

Initiation was found to be normal in all of the LS mutants examined, except LS −15/−26, −19/−29, and −25/−34. These mutants show a number of less intense bands in the region between +1 and −10. Therefore, it is likely that the initiation of transcription in these mutants is affected.

A second minor initiation at −26 identified by Mathis et al. (31) was also detected in these experiments, except in the case of LS mutants −4/−14 to −19/−29. This is probably due to the mismatches of the sequences present in the RNAs synthesized by these mutants and the wt probe. Bands corresponding to this initiation were detected for these LS mutants when the probes were used from the corresponding LS mutants (Fig. 4B). The major RNA start sites (at +1) for the remaining LS mutants were identical to that of wt plasmid (data not shown).

Induction of Transcription of LS Mutants by the EIA Gene. Since almost every nucleotide of the EIA-E promoter is mutated in the present series of LS mutants, they provide us with an opportunity to examine whether or not this promoter contains sequence elements specific for the trans-activation by the viral EIA gene product. HeLa cells were cotransfected with LS mutants and an EIA-containing plasmid (pCD2). CAT activity of the cotransfected cells was quantitated as described (25). The LS mutants in the absence of EIA gene showed the expected pattern, whereas CAT activity for all of the LS mutants was found to be induced by the EIA gene, including those whose transcription was affected severely (Table 2; autoradiogram not shown). Although the degree of induction for a single mutant varied from experiment to experiment, each mutant showed efficient induction (about 4-fold) on at least two occasions. In sum, our results indicate that there is no specific nucleotide sequence upstream from the cap site in EIA-E promoter individually required for the induction of transcription by the EIA gene product.

DISCUSSION

Within 86 nucleotides required for Ad EIA-E promoter function (refs. 33 and 34 and this report) we have identified two specific transcriptional control sequences. One located between −29 to −21 seems to control initiation. Its sequence, 5' C-T-T-A-A-G-A-G-T-T 3', is partially homologous and may be functionally analogous to the TATA box. Numerous studies have established the function of the TATA box in positioning the RNA start sites. It is possible that the difference of nucleotide sequences seen in this case may be an evolutionary variation that occurred during viral evolution with no additional mechanistic significance. Alternatively, polymerase II may require additional viral and/or cellular factors for the RNA chain initiation from this control sequence. Further experiments are needed to resolve this question.

The second control region of the EIA-E promoter maps between −82 and −66. The sequence of this control element is 5' T-G-G-A-G-A-T-G-A-C-G-T-A-G-T-T-T 3', which is relatively pure rich. An exactly homologous sequence has not been seen in other Ad promoters or several eukaryotic polymerase II promoters (19). However, a part of this sequence, 5' T-G-G-A-G-A-T-G-A-C', does resemble very closely the consensus sequence 5' G-A-A-G-A-A-G-A-C' identified by Hearing and Shenk in a variety of transcriptional control regions, including EIA and EIV (16). Another part of this control region, 5' G-T-A-G-T-T-T 3', also shows homology with a consensus sequence, 5' A-C-G-G-G-C-G-T-A-C-G-T-T-T-C 3', identified by Weeks and Jones (19) that is found between −85 and −60 of the EII and EIV promoter sequence.

Recently, Imperiale and Nevin (34) showed that the DNA sequences from −262 to −21 of the EIA-E promoter can function independent of distance or orientation. In their studies they relocated this DNA fragment on the EIA gene that contained 28 bp upstream from the cap site. Present
studies show that control region I is located within 29 bp from the cap site. Furthermore, both of the control regions are essential for transcription because mutations in region I or II reduce transcription drastically. It is conceivable that region I can function only in conjunction with the cap site, whereas region II can function from different locations. Upstream control elements have in general have been shown to be required for the efficient transcription of polymerase II promoters (3–12). It is speculated that the upstream G+C-rich control region found between −100 and −50 of the herpes thymidine kinase gene functions to facilitate the entry of RNA polymerase II (6). Perhaps, control region II of the EIIA-E promoter is similar, facilitating the entry of RNA polymerase II, which then may interact with region I to initiate transcription.

The second part of these studies relates to the role of the DNA sequences in trans-activation by the viral EIA gene product. Almost all of the bases of the EIIA-E promoter were mutated in the present set of LS mutants. In spite of this, EIA induced CAT activity in all of the LS mutants. A simple interpretation of these results is that the EIA gene product induces transcription of the EIIA-E promoter in a sequence-independent manner. However, our data do not rule out the possibility that EIA may effectively recognize more than one such sequence. Our data also do not rule out a possibility of regulatory sequences specific for the EIA present between +68 and +1. But, a LS mutant with mutations of bases between +39 and +29 was efficiently induced by the EIA gene product (unpublished observations).

EIA activates several Ad early promoters (22, 24) and heterologous promoters such as β-globin (35), heat shock gene (36), rat preproinsulin (37), and simian virus 40 early promoter (38). EIIA-E promoter is also activated by the pseudokaryons IE gene (39). All of the above data strengthen the notion that the EIA-mediated trans-activation of these various promoters may not be based on nucleotide sequence specificity. A recent study of the herpes simplex virus glycoprotein D gene promoter also failed to identify sequences specific for its trans-regulation by the viral IE gene (40). Two different mechanisms have been postulated for the mechanism of EIA action: (i) EIA interacts and alters the host cell to facilitate transcription from viral promoters (24, 39) and (ii) EIA catalyzes the assembly of the viral DNA into a stable transcriptional complex (41). Our finding that no specific nucleotide sequence is required for the EIA action fits either of the two hypotheses.

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