Activation of the Adenovirus EIIa Late Promoter by a Single-point Mutation Which Enhances Binding of Transcription Factor IID*

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We have converted the TACAAA sequence present at position −29 of the adenovirus EIIa late promoter into a canonical TATA box by oligonucleotide-directed mutagenesis. When linear templates were analyzed in nuclear extracts, transcription of the mutant promoter showed a 10-fold higher level of activity than that of the wild-type promoter. This increase was correlated with an increased affinity of the mutant promoter for transcription initiation factor IID. Further analyses demonstrated that the activating functions of three EIIa late upstream promoter elements (Huang, D.-H., and Roeder, R. G. (1988) Mol. Cell. Biol. 8, 1906–1914) were maintained in the mutant promoter background. These observations indicated, first, that the upstream elements did not act merely to overcome a rate-limiting initiation step imposed by an inefficient TATA element and, second, that the strength of the interaction between transcription initiation factor IID and the TATA box was directly related to promoter activity.

Increasing evidence suggests that transcription of mRNA-encoding genes in higher eukaryotic cells may be controlled both by basic promoter elements and by other auxiliary (upstream elements and enhancers) elements (1–3). The TATA box, located about 30 base pairs upstream of the initiation site in many genes (1), was shown to be a critical element of the basic promoter (4–6). Furthermore, factors that are necessary and sufficient for accurate in vitro initiation from basic promoters have been identified (7–9), and their roles in the assembly of the preinitiation complexes have been investigated (8, 10–14). Of special note is transcription factor IID (TFIID),1 which has been shown to bind independently of other factors to the TATA region of several promoters (14, 15).2 Factors that interact with upstream elements and enhancers have also been identified; and in several cases, in vitro functions have been demonstrated for the isolated factors, including those for the SV40 early promoter (2), the Drosophila hsp 70 promoter (16), and the adenovirus major late promoter (14, 17, 18). Although little is known about the mechanisms involved in the function of the upstream binding factors, a cooperative interaction between TFIID and the adenovirus major late promoter-specific upstream factor has been demonstrated (14).

Transcription of the gene encoding the 72-kDa DNA-binding protein of adenovirus (EIIa) is controlled by two promoters. The promoter located at map unit 75 is expressed early in infection, whereas the promoter located at map unit 72 is expressed after the onset of DNA replication (19, 20). We have previously found that in nuclear extracts, the EIIa late (EIIaL) promoter is transcribed more efficiently from circular templates than from linear templates (21). The effect of the template topology is dependent upon two distal upstream elements located near positions −265 and −147. In addition, another upstream element located near position −116 appears to be important for transcription of both circular and linear templates, although deletion of this element causes a more profound reduction in transcription of circular templates. Unfortunately, further analysis of the mechanisms by which these elements potentiate transcription of the EIIaL promoter is made difficult by the fact that this gene is poorly transcribed in nuclear extracts. We have also shown that the TACAAA sequence located at position −29 of the EIIaL promoter does function independently as a weak promoter, and we have further speculated that the low activity (relative to promoters with a TATA consensus sequence) results from the presence of the C residue at the third position of this sequence.

In this report, we demonstrate that the transcription of the EIIaL promoter can be activated through an enhanced promoter-factor interaction by the reconstruction of a canonical TATA box. Furthermore, we are able to show that the positive functions of three upstream elements are preserved in this mutant.

MATERIALS AND METHODS

Enzymes and Reagents—Most restriction endonucleases, T4 ligase, Klenow enzyme, polynucleotide kinase, and S1 nuclease were purchased from Bethesda Research Laboratories. Asp-718 and calf intestine phosphatase (molecular biology-grade) were purchased from Boehringer Mannheim. [α-32P]dATP and [α-32P]GTP were purchased from Du Pont-New England Nuclear.

Construction and Identification of Point Mutation—The method used for oligonucleotide-directed site-specific mutagenesis was essentially as described by Zoller and Smith (22). The M13 clone used for mutagenesis contained the EcoRI/HindIII fragment that extends from positions −418 to +330 of the EIIaL promoter. The 20-mer contained the DNA sequence from positions −57 to −18 of the antisense strand, except for the desired alteration at position −27 (Fig. 1). In order to enrich the yield of mutants, two cycles of mutagenesis were carried out under the same conditions, except that hybridization was performed on ice for 1 h for the first cycle and at 33 °C for 30 min for the second cycle. Plaques obtained from the second cycle of
mutagenesis were screened with end-labeled 20-mer on triplicate filters. These filters were hybridized under identical conditions (25 °C), but were later washed at three different temperatures (35, 45, or 55 °C). Approximately 10% of the plaques were positive after washing at 55 °C.

The positive plaque was purified and used for the preparation of M13 replicative form DNA. The EcoRI/HindIII insert carrying the presumptive mutated EIIaL gene was cloned into the EcoRI/HindIII sites of pUC13. This plasmid clone was cleaved at a unique Asp-718 site and labeled by polynucleotide kinase. The purified Asp-718/HindIII fragment was then sequenced by the method of Maxam and Gilbert (23), which verified that the C residue present at position −27 (in the parental clone) was mutated into a T residue (designated CT-27).

Construction of Deletion Mutants—Clone CT-27 was used for subsequent construction of deletion mutants. The restriction sites used for deletion were SstI, BalI, and DdeI sites located at positions −223, −133, and −34, respectively. The 5′ end generated by SstI digestion was cloned into the SstI site of pUC13. The 5′ ends generated by BalI and DdeI digestions were cloned into the Smal site of pUC13. All the 3′ ends of these inserts were generated by EcoRI digestion and were cloned into the EcoRI site.

In Vitro Transcription and RNA Analysis—The standard procedures used for in vitro transcription in HeLa nuclear extracts and for transcription analysis by run-off and S1 nuclease assays were previously described (21).

DNase I Footprinting Analysis—The TFIIID fraction used for this analysis was prepared from a single-stranded DNA-agarose column following a chromatographic scheme which includes phosphocellulose, DE52, aminooctyl-agarose, and fast-protein liquid chromatography (Mono Si). EIIa late promoter DNA containing either wild-type promoter (deletion mutant at position −131) or mutant promoter (deletion mutant at position −133) was digested by BamHI and labeled at the 5′ end by [α-32P]dATP and Klenow enzyme. The end-labeled DNA was then digested with EcoRI and isolated by agarose gel electrophoresis. Varying amounts of the TFIIID fraction were incubated with 1 ng of probe (about 15,000 cpm) and optimal concentrations of poly(dG-dC) at 4 °C for 10 min under standard transcription conditions (except for the absence of nucleoside triphosphates). Following a further incubation at 30 °C for 30 min, digestion was carried out at 30 °C for 30 s in the presence of 0.6 μg/ml DNase I. Reactions were terminated by the addition of 3 μg of TRNA and EDTA to a final concentration of 8 mM. After extraction with phenol and chloroform, purified DNA fragments were analyzed on a sequencing gel (23). Autoradiography was performed at −70 °C with an intensifying screen.

RESULTS

Construction and Run-off Transcriptional Analysis of Point Mutant—An oligonucleotide-directed site-specific mutagenesis protocol (22) was used to convert the TACATA box sequence of the EIIaL promoter into the canonical TATAAA sequence. A derived mutant was verified by sequence analysis to contain the expected C-to-T transition at the third nucleotide of the TACATAA sequence and was designated CT-27 (Fig. 1).

The phenotype of CT-27 was first compared to that of the wild-type promoter in a run-off transcription assay (Fig. 2). The transcript size expected for accurate initiation (at the natural cap site) in the EcoRI-cleaved template was 330 nucleotides. For the wild-type promoter, a weak band corresponding to the correct run-off transcript was observed over a wide (6-fold) range of template concentrations (lanes 1–6); in contrast, a mutant promoter gene generated, over the same template concentration range, a 10-fold higher level of the same transcript (lanes 7–12). Thus, in linear templates, the mutant EIIaL promoter is more actively transcribed than is the wild-type promoter.

Transcription Analysis of Circular Versus Linear Templates—We have previously shown that transcription of the wild-type EIIaL promoter is significantly higher in circular templates than in linear templates (21). To determine whether transcription of the mutant EIIaL promoter was also enhanced in the circular template, transcripts from both circular and EcoRI-cleaved linear templates were analyzed by the S1 nuclease mapping technique. As shown in Fig. 3, the circular wild-type template was much more active than the linear wild-type template. Similarly, the circular CT-27 template was also more actively transcribed than the linear CT-27 template. However, the magnitude of the topology-dependent enhancement was not as profound for CT-27 (5-fold) as that observed for the wild-type promoter (10-fold).

Transcription Analysis of Upstream Deletion Mutants—The topology-dependent transcriptional enhancement in the wild-type promoter was previously shown to require two distal upstream elements located near positions −265 and −147 (21). To determine whether these elements were also important for maximal transcription of the circular CT-27 template, upstream deletion mutations constructed in CT-27 were analyzed. As shown in Fig. 4, transcription of the circular template was reduced in a stepwise fashion in both the −233 and −133 mutants. In contrast, deletion from positions −418 to −133 had no effect on transcription of linear templates. Therefore, the wild-type functions of these two elements were still preserved in CT-27, although the negative effect of each deletion (2-fold in each case) was not quite as great as that.
observed for the wild-type promoter (3-fold in each case). Thus, the cumulative effect of these two elements in potentiating transcription of the circular template of CT-27 should be about 4-5-fold. This is consistent with the earlier observation that the transcription of the circular template was enhanced about 5-fold for CT-27.

Previous studies also showed that the deletion of an element located at position -116 reduced transcription of the wild-type promoter in both circular and linear templates, but that the effect was much greater with the circular template. A similar situation was also observed with the mutant promoter. As shown in Fig. 4, the deletion of DNA sequences between positions -133 and -34 caused more than a 10-fold reduction in the transcription of the circular template, but only a 3-fold reduction in that of the linear template. Thus, it appears that the wild-type function of this proximal upstream element is also preserved in CT-27.

Enhanced TFIID-TATA Box Interaction—The linear form of the -34 deletion mutant derived from CT-27 was still transcribed 10-fold more efficiently than that of the corresponding deletion mutant derived from the wild-type promoter; although the data showing a direct comparison are not given here, the same conclusion can be deduced from results presented in Fig. 3 and 4. Thus, the observed increase in transcription of the EIIaL gene in CT-27 appeared to result primarily from an increase in the strength of the basic promoter. Previous studies have indicated that TFIID both binds to the TATA region of the adenovirus major late promoter (14) and is required at an early step in the formation of preinitiation complexes (8, 10, 11, 21, 22). Alterations in this interaction could be one of the major parameters which control the strength of a given promoter. Therefore, we examined the ability of TFIID to bind wild-type and mutant promoters by DNase I footprinting analysis (24). As shown in Fig. 5 for the mutant promoter, the region between positions -18 and -37 was almost fully protected from digestion when the maximal amount of TFIID was used (lane 7). However, protection over the same region was barely observed for the wild-type promoter. With serial increments in the TFIID concentration, we estimated that the wild-type promoter required about 8-fold more TFIID than did the mutant promoter for the same extent of protection (compare lane 7 for wild type and lane 4 for mutant type). It is thus obvious that the alteration of the EIIaL promoter sequence has a direct effect on the affinity for TFIID. Moreover, there was a good correlation between the increased affinity for TFIID and the increased level of transcription when comparing the mutant versus wild-type templates.

DISCUSSION

An interesting feature of the adenovirus EIIa late promoter is the presence of three positive cis-acting upstream elements whose functions are totally or partially dependent upon a particular template topology (circularity) (21). In order to understand the mechanisms by which these upstream elements potentiate transcription, it is necessary to identify and characterize factors that interact with these elements. Since...
FIG. 4. Effect of upstream deletion on transcription of mutant promoter. A, diagram showing restriction sites used for the construction of deletion mutations. The 5' ends generated by SstI digestion were cloned into the SstI site of pUC13. The 5' ends generated by BglII and DdeI digestion were cloned into the SalI site of pUC13. B, autoradiograph showing S1 nuclease analysis of in vitro transcripts obtained from circular and linear templates of upstream deletion mutants of CT-27. The end points of the deletion mutants are indicated on the top of the gel.

this gene is transcribed poorly in nuclear extracts, one objective has been to obtain a more sensitive assay system. For this purpose, we chose to mutate the DNA sequence of the minimal promoter element with the expectation that the generation of a canonical TATA box might activate the weak promoter in a fashion which preserves the functions of the upstream elements. An alternative possibility was that such a mutation might maintain the normal level of activity observed with the wild-type promoter, but render it independent of the upstream elements (if, for example, the function of the upstream elements was simply to overcome a rate-limiting step imposed by an altered interaction at the noncanonical TATA element). A clear-cut distinction between these possibilities could thus provide significant information about the mechanism of action of the upstream elements.

We have shown that transcription of the EIIa late gene in vitro was stimulated about 10-fold by a C-to-T transition at the third nucleotide of the normal TACAAA sequence. In addition, we have shown that the deletion of the proximal upstream element (around position -116) in the mutant promoter, as observed for the wild-type promoter, reduced transcription of circular templates more severely (about 10-fold) than that of linear templates (about 3-fold), indicating that the normal function of this element was preserved. Similarly, deletions of each of the two distal elements (near positions -265 and -147), whose effects were seen only with circular forms of the wild-type template, markedly reduced transcription of circular (but not linear) mutant templates. The overall effects of template topology were slightly reduced in mutant templates (5-fold increased activity with a circular template) relative to wild-type templates (10-fold increased activity with a circular template) and largely reflected a slight decrease (from 3- to 2-fold) in the contribution of each of the two upstream elements. However, these minor quantitative differences might well have reflected limiting concentrations of factors in nuclear extracts, when mutant templates were transcribed. Thus, since transcription of the linear mutant template was as efficient as that of the circular wild-type template, a uniform 10-fold increase in transcription of circular mutant templates might require a proportionally higher level of one or more of the general transcription factors and/or the factors that interact with the distal upstream elements. Despite the minor quantitative differences, the most significant finding is that the functions of these distal upstream
elements were still evident and dependent upon template topology in the promoter. As discussed earlier, two fundamentally different mechanisms might potentially account for the action of the upstream elements (see above). However, the observation that the functions of all three upstream elements (one proximal and two distal) were not dependent upon the level of activity of the basic core promoter suggested that these elements operate independently to facilitate transcription. Thus, they apparently do not function simply to overcome a rate-limiting step imposed by a noncanonical TATA sequence whose factor interactions (below) may be impaired. A practical implication of this result is that this mutant should greatly facilitate future studies of these upstream elements and interacting factors. Since position -30 of a number of genes contains A+T-rich sequences which differ from the canonical TATA box only by one nucleotide (1), it is possible that these sequences may also result in weak promoters and that the approach described above will be useful for the activation and analysis of these promoters.

With respect to the function of the TATA element, it has been shown that transition or transversion mutations at the third nucleotide of the TATA box result in promoter down-regulation (34). The observation that the EIIaL promoter is activated by a point mutation at the third nucleotide of the TACAAA sequence is consistent with the notion that the C-to-A mutation also reveals a rate-limiting step imposed by a noncanonical TATA sequence whose factor interactions (below) may be impaired. A practical implication of this result is that this mutant should greatly facilitate future studies of these upstream elements and interacting factors. Since position -30 of a number of genes contains A+T-rich sequences which differ from the canonical TATA box only by one nucleotide (1), it is possible that these sequences may also result in weak promoters and that the approach described above will be useful for the activation and analysis of these promoters.

The observation that the EIIaL promoter is activated by the C-to-A point mutation also reveals a rate-limiting step imposed by the noncanonical TATA sequence of the wild-type promoter. This limiting step might be any one of the several distinct steps involved in the assembly of stable preinitiation complexes (8, 10, 12, 13), one of which involves the direct interaction of a ubiquitous factor (TFIID) with the TATA element (14). An alternative possibility is that one of the subsequent steps, such as initiation, is limited as a result of an unfavorable TFIID-TATA elements interaction. Whatever the limiting step, it could result from either quantitative or qualitative changes in the TFIID interaction(s), affecting either the number of functionally active preinitiation complexes or the rate of initiation upon such complexes. Although these possibilities have not been distinguished, our studies have shown that the C-to-A mutation results in an 8-fold increase in the affinity of TFIID for the promoter. This correlates well with the 10-fold increase in the transcription observed in vitro, indicating that altered TFIID binding is directly related to transcriptional activation in the mutant promoter. It is not yet known, however, whether the increased TFIID binding reflects an increased rate of binding or a decreased rate of dissociation. It may be noted that the limited DNase protection effected by TFIID on the EIIa late promoter (positions -18 to -37) contrasts markedly with the broad region of TFIID interaction on the adenovirus major late promoter (14). However, more recent studies of other cellular and viral promoters have shown that TFIID interactions are restricted to the upstream region. Other studies have shown that downstream interactions and subsequent recognition by RNA polymerase II and other basic factors may be facilitated by upstream regulatory factors. Thus, the TFIID interaction pattern observed for the EIIa late promoter is not unusual and may also be altered in response to upstream factors.

The presence of stable (pre)initiation complexes which need not be totally reformed during repeated rounds of transcription (11) allows for the possibility of multiple control elements which, like the TACA and the upstream elements analyzed here, may have independent additive effects upon the overall level of transcription. With respect to the limitation imposed by the noncanonical TATA element, it is also worth noting that the interaction of the adenovirus major late promoter-specific factor with its upstream target sequence (and presumably its function) is greatly facilitated by the concomitant binding of TFIID to the adjacent TATA element, indicative of a direct interaction between these two factors (14). If there is a similar interaction between TFIID and factors binding to the EIIaL upstream elements, then our studies suggest that it is not this aspect of TFIID function which is impaired in the wild-type promoter. Since the rate-limiting step in question here may also exist for other promoters which do not contain consensus TATA sequences and since a detailed analysis of this step may facilitate the understanding of the parameters that can potentially affect the assembly and function of transcription complexes in general, it will be advantageous to use the EIIaL promoter as a model system for the identification and further investigation of this step.

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REFERENCES


3 M. Horikoshi, unpublished results.