Generation and functional analyses for base-substitution mutants of the adenovirus 2 major late promoter

Yie-Teh Yu and James L. Manley

Department of Biological Sciences, Columbia University, New York, NY 10027, USA

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ABSTRACT

The function of guanosine residues surrounding the TATA box of the adenovirus 2 major late promoter (MLP) in promoting efficient transcription initiation and in selecting a specific transcription start site *in vitro* has been examined. Multiple and single base substitutions (G→A) were generated in this region (from -63 to +25 relative to the cap site, +1) of the MLP. The promoter activities of the wild type and 21 mutants were assayed in an *in vitro* transcription system using whole cell extract (WCE) prepared from HeLa cells. The results suggest that the strings of G residues immediately adjacent to the TATA box are not required for full promoter activity *in vitro*. These G residues also appear not to be involved in the selection of a specific transcription start site by RNA polymerase II *in vitro*, since the identical cap site was used by wild-type and mutated MLP's. However, two G residues (-55 and -57) were identified as part of an upstream promoter element: a G→A transition at either -55 or -57 resulted in a 2.8 fold reduction in promoter activity. However, neither single nor double G→A transitions at -62 and -63 had an effect on promoter activity.

INTRODUCTION

The structure of promoters for animal cell RNA polymerase II has been the focus of much study in recent years (for review, see (1)). One promoter element, the TATA box, is located approximately 26-34 base pairs (bp) upstream from the RNA start site in most cellular and viral genes (1,2), and has been shown to be required for efficient and/or accurate transcription initiation both *in vitro* and *in vivo* (3-8). In addition, nucleotide sequences that lie further upstream, but which are not as well conserved as the TATA box, frequently play important roles in determining the efficiency of transcription initiation (9-12). In many cases, these sequences are more crucial for promoter activities *in vivo* than they are *in vitro* (10,11).

The adenovirus 2 major late promoter (MLP), which is more efficiently utilized *in vitro* than are other pol II promoters (13, our unpublished results), has been used as a prototype pol II promoter. Studies using deletion mutants suggested that nucleotide sequences within 15-17 nucleotides on either side of the TATA box are required to obtain optimal levels of transcription initiation *in vitro* (3,5). Point mutations in the TATA box of the MLP resulted in 2-5 fold reductions in promoter activity *in vitro* (14).

However, the question of whether nucleotide sequences adjacent to the TATA box also contribute to promoter strength and/or to the selection of transcription start sites has not been addressed. The MLP is somewhat unusual in that it contains two clusters of G residues flanking the TATA box (15). It has been tempting to speculate that one or both of these G stretches contribute to the strength of the MLP (eg,16,17). Studies on the silk fibroin and Herpes Simplex virus thymidine kinase promoters, using DNA templates containing point mutations in this region, have suggested a possible function for nucleotide sequences surrounding the TATA box (8,18). In addition to the TATA box, an upstream promoter segment of the MLP has also been revealed by recent *in vitro* and *in vivo* studies, although its location has not been determined precisely (9,12,19). Based on a homology with the rabbit β -globin promoter, the nucleotide sequences between -59 and -69 have been proposed to constitute at least part of this upstream promoter segment (9). In this paper, we describe the generation of mutants with multiple or single base substitutions (G→A) in this region and report the effects of these mutations on the efficiency and specificity of transcription initiation *in vitro*. Although the G residues surrounding the TATA box were found not to be important in promoter function, nucleotides contributing to an upstream promoter element were identified.

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased either from International Biotechnology, Inc. (New Haven, Conn.), or from New England Biolab (Beverly, Mass.). The assay conditions suggested by the companies were followed for restriction enzyme digestion reactions. Hela whole-cell lysate was prepared and used as described previously (20,21).

Generation of base substitution mutants

Plasmids pXB210 and pXB205, which contain portions of the Ad2 MLP from -66 to +193 and from +17 to +193, respectively (5), were cut with HindIII and Eco RI to excise the Ad2 inserts. The viral DNA's were then recloned into M13 phage mp9 RF DNA(22), resulting in clones mp9-210 and mp9-205. To generate G→A transitions in the *l* strand of the adenovirus insert (from approximately -66 to + 17), a gapped double-stranded DNA was formed by hybridization of 1.5 μ g circular mp9-210 phage DNA and 2.0 μ g Eco RI linearized mp9-205 RF DNA in 20 μ l of 10 mM Tris, pH 8.0, 1 mM EDTA and 0.2 M NaCl, according to Everett and Chambon (23). Following denaturation of the DNA's by immersion in a boiling water bath for 3 min, hybridization was carried out at 67°C for 3 hrs. The mixture was then allowed to cool slowly to room temperature and the DNA was precipitated with ethanol. The gapped DNA was redissolved in 1.5 mM Na-citrate, pH 7.0, 15 mM NaCl and treated with 3 M sodium bisulfite at 37°C for 70 min (24). The

reaction was terminated by dialysis exactly as described by Shortle and Nathans (24). After dialysis, the DNA was precipitated with ethanol, redissolved in 10 mM Tris, pH 8.0, 1 mM EDTA, and transfected into *CaCl*₂-treated *E. coli* cells (ung⁻, BD 1528, a gift from D. Shortle) using *E. coli* 71.18 cells as the bacterial lawn during transformation (25). Mutants were identified by nucleotide sequence analyses of purified phage DNA's using 'dideoxy' sequencing procedures (22).

Construction of mutants with single or double base substitutions from mutants with multiple mutations

Mutants 5 and 26 were used to construct mutants with single base substitutions at -57 and at -55, respectively (Table I). Mutant 19 was used for the construction of the mutant with double point-mutations at -62 and -63. For experimental details, see the legend to Figure 3.

Quantitative analyses of wild-type and mutated MLP activities *in vitro*

RF DNA's of wild-type MLP (mp9-210) and mutants were digested to completion by Taq I. The promoter activities of mp9-210 and each mutant were assayed by measuring the synthesis of specific run-off transcripts in a whole cell lysate transcription system (21) using α -³²P-GTP as the radioactive label. The ³²P-labeled RNA transcripts were treated with glyoxal and analysed on 1.4% agarose gels (26). The total DNA concentration in each assay was 48 μ g/ml. Dried gels were exposed to x-ray films (Kodak XAR-5 or SB-5) at room temperature. The relative transcriptional efficiency of each promoter was determined by scanning the autoradiograms at 800 nm and then comparing each peak area corresponding to the ³²P-labeled run-off transcript with that of mp9-210 (transcriptional efficiency = 1). In the experiments that included Sal I truncated pXB210 DNA, 9 μ g/ml of pXB210 DNA was mixed with 40 μ g/ml of Taq I cut RF DNA's.

Mapping of the 5'-ends of RNA transcripts synthesized *in vitro*

Unlabeled run-off RNA transcripts from Taq I-truncated RF DNA's of mp9-210 and each mutant were synthesized in WCE as described before. Localization of the 5'-ends of RNA transcripts was carried out by primer extension analysis (18) using a 5'-end labeled single-stranded DNA primer encompassing nucleotides from +83 to +34.

RESULTS

Generation of MLP mutants with base substitutions (G→A)

Earlier *in vitro* studies indicated that the major late promoter deletion mutant pXB210, which contains wild-type nucleotide sequences to -66, displays a promoter activity similar to a wild-type MLP clone (containing sequences up to -400) (5). Therefore, we chose pXB210 as a starting material, and generated G→A transitions between -66 and +17 (Fig. 1). The nucleotide sequences of 21 mutants were identified and the

Figure 1.

Construction of base substitution mutations (G→A) in the MLP. Clones mp9-210 and mp9-205 contain portions of Ad2 DNA from -66 to +193 and from +17 to +193, respectively, inserted at Eco RI and Hind III sites in M13 phage mp9 (22). To generate G→A substitutions in the *l* strand of the cloned adenovirus DNA segment, a gapped double-stranded DNA was formed by hybridization of circular mp9-210 phage DNA and Eco RI linearized mp9-205 RF DNA. The gapped DNA was then treated with 3M sodium bisulfite for 70 min and transfected into *CaCl*₂-treated *E. coli* cells (BD1528, *ung*⁻). The mutants were screened by nucleotide sequence analyses of purified phage DNA's.

base substitutions of each mutant are presented in Table 1. The nucleotide sequence of the wild-type promoter (mp9-210) is shown at the top of the table. Base substitutions were also found in the regions just downstream from +17. They were likely due to mismatches in this region during the formation of gapped DNA.

Effect of multiple G→A transitions on MLP activities *in vitro*

The promoter activities of mp9-210 and the mutants derived from it were assayed for their ability to direct the synthesis of specific run-off transcripts in a whole cell lysate transcription system (21). The results of two sets of transcriptional assays are

Table 1.

Nucleotide sequences and relative *in vitro* transcriptional activities of MLP mutants. The nucleotide sequences of the wild-type late promoter in mp9-210, from -86 to +25, are shown at the top of the table. The TATA box, the RNA cap site and two G residues at -55 and -57 are underlined. Only the G→A point mutations in each mutant are shown. The level of transcription from each mutant was determined by scanning the autoradiograms shown in Fig. 2 as described in Materials and Methods.

NUCLEOTIDE SEQUENCES AND RELATIVE TRANSCRIPTIONAL ACTIVITIES OF MLP MUTANTS												
	-70	-60	-50	-40	-30	-20	-10	+1	+10	+20		
W.T.	GAATTCCTAGGCCACGT	ACCGGGT	CTTCCTGA	AGGGGGC	<u>TATAAA</u>	GGGGGT	GGGGGC	CGTTCGTCCTCA	CTCTCTTCGCATC	GCTGTCTGAGG		
MUTANT NUMBER												RELATIVE TRANSCRIPTIONAL ACTIVITY
I. NO SIGNIFICANT EFFECT												
1												0.86
16				A	A A A		A A					0.76
18		A					AAA		A			1.02
19		AA		A	A A A		A A A					1.19
25		A			A AAA		A A A A			A		0.89
28			A		A A A		AAA A A					0.94
30					A A AA							0.91
34			A		A		A					0.86
35				A			AAA A A					0.95
37		A					A A					0.87
39					AA		A A			A		1.07
43		A					A A A					0.95
46							A			A A A		0.81
47			AA		A A		A A			A A		0.83
II. DOWN MUTATIONS												
5			A			A		A A			A A	0.43
10		A	A		A A		AA A A		A A			0.27
22		A	A		A A					A		0.45
26			A				A A				A	0.24
31		AA	A		A A		A					0.27
33			A		A A							0.39
49			A			A						0.29
III. SINGLE OR DOUBLE MUTANTS AND THEIR COMPLEMENTARY MUTANTS												
55			A									0.38
57												0.40
6263		AA										1.08
26455							A A				A	0.82
5457						A	A A				A A	1.05

shown in Fig. 2. The arrowhead a points to the specific RNAs transcribed from Taq I-truncated mp9-210 and a number of mutants. Several mutants displayed lower transcriptional efficiencies than that of mp9-210 (mutants 10, 26, 31 in Fig. 2A; mutants 22 and 30 in Fig. 2B). In order to demonstrate that the lower transcriptional efficiencies of the mutants shown in Fig. 2 were not artifactual, i.e., due to lower recovery of labeled transcripts, Sal I-truncated pXB 210 was included in the incubation mixture as an internal standard at a molar ratio of mp9-210 to pXB 210 of 4:1. Interestingly, under these conditions, the wild-type promoter in pXB 210 was utilized more efficiently in the presence of the down mutants than in the presence of either mp9-210 or mutants with similar promoter activities, as indicated by the different levels of labeled transcripts from pXB 210 templates (arrowhead b, Fig. 2). These observations, which were obtained in several independent experiments, not only indicate that the lower

Figure 2.

In vitro transcription of the wild-type MLP and mutants. RF DNA's of mp9-210 and mutants were digested with Taq I and used for *in vitro* transcription assays. In the assays shown on the right panels, Sal I-truncated pXB 210 DNA was included in each incubation mixture as an internal standard. Run-off transcripts synthesized were extracted, denatured with glyoxal, and resolved on a 1.4% agarose gel. Autoradiograms of two different experiments are shown in A and B. The mutant analyzed is indicated below each lane. The arrowhead a indicates the run-off transcripts synthesized from Taq I-truncated mp9-210 and mutants, and arrowhead b indicates the run-off transcripts synthesized from Sal I-truncated pXB 210 DNA. M: RNA size markers; wt: wild type (mp9-210).

transcriptional efficiencies of down mutants were real, but also suggest that the down mutants compete less effectively than does the wild type for a required factor in the extract. The relative transcriptional activity of each mutant was determined as described in the methods section and is listed in Table 1.

Promoter activities were also assayed at lower DNA concentrations (16 $\mu\text{g}/\text{ml}$), because earlier studies had revealed greater differences in promoter activities under such conditions (9,19). However, the relative transcriptional activities of mp9-210 and the mutants derived from it remained the same under these assay conditions (results not shown). When the nucleotide sequences of these mutants were compared, it was

Fig. 3 Construction of MLP mutants with single or double point mutations.

Figure 3.

Construction of mutants with single or double substitutions from mutants 5, 19, and 26. RF DNA's of mp 9, mp9-210, mutants 5, 19, and 26 were digested by Bgl I and Hind III. The resulting 7.05 kb fragment from mp 9 was gel purified (32) and used as a vector for the new mutants. The 410 bp Bgl I-Hind III fragments from mp9-210 and the mutants were then digested with Hpa II (-52 of MLP), and the resulting 180 bp and 250 bp fragments were separated by 7% polyacrylamide gel electrophoresis and eluted (33,34). To construct the new clones, the purified 7.05 kb Bgl I-Hind III fragment from mp 9 was ligated to the appropriate 180 bp and 250 bp fragments as described in the figure. Each mutant was identified by nucleotide sequence analysis of phage DNA. B: Bgl I; H: Hind III; R: Eco RI; h: Hpa II; L: ligase.

clear that the G residues located either downstream or upstream from the TATA box were not required to obtain full promoter activity *in vitro*.

An upstream element of the late promoter

As shown in Table 1, all of the mutants with reduced promoter activities contain a G→A transition at either -55 or -57. To determine whether these two G residues are indeed required to obtain wild-type levels of transcription, mutants with single base changes at -55 (mutant 55) and at -57 (mutant 57) were constructed from mutant 26 and mutant 5, respectively (Fig. 3). To demonstrate further that only the G→A mutations at -55 or -57 are required for the observed reduction of promoter activity, MLP mutants complementary to mutant 55 and mutant 57, 26d55 and 5d57, respectively, were constructed. Mutants 26d55 and 5d57 contain all the mutations in mutant 26

Figure 4.

In vitro transcription of single and double base substitution mutants. The same experimental procedures as described in Fig. 2 were followed. The mutant analyzed is shown below each lane. wt: wild type (mp9-210).

and mutant 5, respectively, except at positions -55 and -57, which are replaced by wild-type sequence. The promoter activities of these mutants were assayed *in vitro* and compared with the promoter activities of other down mutants and with the wild-type promoter. As shown in Fig. 4, mutant 55 displays a similar promoter strength as do the other down mutants that contain G→A transitions at -55. However, the promoter activity of mutant 26d55, which contains wild-type nucleotide sequence in this region, has been restored to the wild-type level. Mutant 57 and mutant 5d57 also displayed the same promoter activities as the down mutant and wild type, respectively (Fig. 4). These observations demonstrate that the two G residues at -55 and -57 are part of an upstream promoter element.

The nucleotide sequences from -59 to -69 have been proposed to constitute an upstream promoter segment of the MLP (9). As shown in Table 1, single G→A transition at -62 or at -63, as well as double G→A transitions at -62 and -63 (mutant 19), do not have detectable effects on promoter activity. This result is further supported by the wild-type level promoter activity of mutant 6263, which contains double base-substitutions at -62 and -63 (Fig. 4). These observations suggest that the two G resi-

dues at -62 and -63 are not an essential part of the upstream promoter element of the MLP *in vitro*.

Mapping of the 5'-ends of RNA transcripts synthesized *in vitro*

It has been demonstrated that the TATA box is required for the selection of specific transcription start sites utilized by RNA polymerase II (6,8). To determine whether the G residues surrounding the TATA box are also important for the selection of specific transcription start sites, the 5'-ends of the run-off transcripts synthesized from the mutants were located by primer extension analyses (18). The run-off transcripts from all the MLP mutants showed the same 5'-ends as did the wild-type clone, mp9-210. The results TATA box are not required for the function of TATA box in fixing the transcription initiation sites. As shown in Fig. 5, doublets were detected in the run-off transcripts. The lower band (83 nucleotide long) corresponds to the extended product of the predicted size if the normal cap site (+1) was used. Earlier studies indicated that wild-type MLP transcripts synthesized in WCE transcription systems initiate from only one site (+1) and are quantitatively capped (19,20). Thus, we believe that the upper band in Fig. 5 is due to an artifact of reverse transcription. These results suggest that the G residues flanking the TATA box are not required for the function of TATA box in fixing the transcription initiation sites.

Discussion

The possible promoter function of the clustered G residues flanking the TATA box of the adenovirus 2 major late promoter has been investigated. These clustered G residues represent the most characteristic feature of the late promoter : 19 out of 39 nucleotides (from -38 to +1) are G residues, including the 11 residues immediately upstream and downstream from the TATA box (see Table 1). Since the late promoter is the most active pol II promoter *in vitro* (13), it was speculated that these abundant G residues might contribute in part to the strength of this promoter. However, our data indicate that these G residues are not required for promoter function *in vitro*. It was quite surprising to observe that drastic changes in the nucleotide sequence in this region had no significant effect on the activity of the major late promoter. The TATA box thus may be the only nucleotide sequence in this region required for efficient initiation of transcription *in vitro*. Since only G→A transitions were analyzed, it is possible that other nucleotide changes in this region might affect promoter activity.

These G residues have also been proposed by others to play a role in promoter function during initiation (16,17). The abundant C≡G base pairs surrounding the TATA box of a number of eukaryotic genes have been postulated to delimit the TATA box as a 'trap' for the transcriptional enzymes to bind to (17). Multiple G→A transitions in this region might then be predicted to destroy such a 'TATA trap' domain, thereby pro-

Figure 5.

Localization of transcription start sites *in vitro*. A. Unlabeled RNAs were transcribed from Taq I-truncated RF DNA's of mp9-210 and mutants in a WCE transcription system. The open block represents the major late promoter from -66 to +193, and the solid line indicates the nucleotide sequences of mp 9. For primer extension analyses, a 5'-end labeled single-stranded DNA primer was purified from a Pvu II-Dde I fragment

(+34 to +83), and reverse transcription was carried out according to McKnight and Kingsbury (18). R.T.: reverse transcription; P: primer. B. The labeled extended primers were analyzed on an 8.3 M urea, 8% polyacrylamide gel (34). The nucleotide size markers were the G and A sequence ladders of mutant phage DNA's, using an M13 primer (15 mer) from New England Biolab (Beverly, Mass.). The numbers on the left of the gel indicate the sizes of the fragments, and the arrowhead MLP(+1) on the right of the gel points to the extended primer of predicted size (83 N) if the normal cap site (+1) was utilized for initiation of transcription. Mutants analyzed are indicated below each lane. m: mock; wt: wild type.

longing the scanning process of the transcriptional machinery for the TATA box. Our data argue against this hypothesis, because multiple G→A transitions in this region (Table 1) failed to reduce the late promoter activity *in vitro*.

The G residues downstream from the TATA box of the late promoter (-24 to -6) have also been suggested to form part of a hairpin structure and as a result facilitate the separation of the template strand during transcription initiation (16). Multiple G→A transitions in this region would disrupt such a hairpin structure, perhaps thereby reducing the transcriptional efficiency. However, since no effect of such G→A transitions on promoter activity was observed, our data suggest that formation of such a hairpin structure downstream from the TATA box is not required for transcription initiation *in vitro*. However, whether the formation of a hair-pin structure in this region is required for transcription initiation *in vivo* remains to be answered.

Earlier studies on the late promoter indicated that upstream sequences from -86 to -51 (19), from -97 to -34 (9), or from -110 to -80 (12) are important for promoter activity *in vitro*. The data of Hen *et al.* (9) suggest either that two sets of sequences are involved or that only one promoter element, centered at -62, is located in this region. Our study identified two G residues (at -57 and -55) that are required for obtaining wild-type levels of transcription. Furthermore, the two G residues at -62 and -63 do not appear to be required for efficient transcription *in vitro*. However, our data do not illustrate whether the late promoter contains one upstream element or two separate elements. If there is only one upstream promoter element, then it should include both the two G residues at -55 and -57 and also some nucleotide sequences further upstream from -63. Although the two G residues at -62 and -63 are not required for transcription *in vitro*, it is likely that other nucleotide sequences in this region may be important for efficient transcription *in vitro*. If two upstream nucleotide sequences are involved in determining the activity of late promoter, one element is likely located between -62 and -51, including the G residues at -55 and -57. The other promoter element may be located further upstream, between -97 and -63. Two upstream promoter elements have been identified in the promoters of the herpes simplex virus tk gene (18) and the rabbit β -globin gene (27) at similar distances upstream from the cap sites. If the major late promoter contains two promoter elements, the

element located further upstream has yet to be identified precisely. Although the two G residues at -62 and -63 are not required for transcription from the major late promoter *in vitro*, recent studies from our laboratory (E.D. Lewis and J.L. Manley, manuscript in preparation) showed that nucleotide sequences between -66 and -60 are required to obtain efficient transcription initiation *in vivo*. Studies on the possible promoter function of the two G residues at -62 and -63 *in vivo* are currently in progress.

It has been shown that a 21 bp fragment containing major late promoter sequences from -32 to -12 is sufficient to promote specific initiation of transcription *in vitro* when it was inserted into pBR 322 (28). However, the transcriptional efficiency of this mutant was 5-10 fold lower than that of the wild type. This observation indicated that the MLP TATA box alone can not account for the high activity of this promoter *in vitro* (13). Our results demonstrate that the abundant G residues adjacent to the TATA box are not required for efficient transcription of the MLP *in vitro*. Other nucleotide sequences that are involved in efficient promotion of transcription *in vitro* may include the upstream promoter element(s) identified in this study and/or the region surrounding the transcription start site (29-31). The nucleotide sequence surrounding the cap site of the MLP has been shown to be required to obtain wild-type levels of transcription (3,5,28, E.D. Lewis and J.L. Manley, manuscript in preparation). However, the exact nucleotides involved have not been identified. The identification of the promoter element(s) in the cap site region and possibly in the further upstream region will shed more light on how the MLP functions.

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