The adenovirus major late promoter TATA box and initiation site are both necessary for transcription *in vitro*

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ABSTRACT

Mutagenized DNA templates and HeLa whole cell extracts were used to study the effects of promoter-specific base changes on *in vitro* transcription. DNA templates where the initiating adenine (+1) was changed to thymidine (AT+1) in the adenovirus 2 major late transcription unit were transcribed with 50% efficiency of the unaltered template. We have described a mutant at the TATA box, where the A at position -28 was changed to a C (AC-28). Transcription efficiency was reduced to less than 20% of control in the AC-28 mutant (Concino *et al.*, 1983, J. Biol. Chem. 258: 8493-8496). Primer extension analysis revealed increased 5' end heterogeneity for *in vitro* transcripts derived from AC-28 and AT+1 DNA templates. Specific transcription was completely abolished from AT+1 DNA templates when a second change was introduced within the TATA sequence, creating a double mutant (AC-28 • AT+1). Neither the AC-28 nor the AT+1 change alone had such an effect, suggesting a coordinated interaction in transcription initiation involving both the TATA box and the initiation site.

INTRODUCTION

RNA polymerase II in eukaryotic cells is responsible for catalyzing the synthesis of mRNA precursors (1). Although this function is not an inherent characteristic of the purified enzyme, accurate transcriptional initiation can be detected *in vitro*, in crude HeLa cell extracts (2), nuclear extracts (3), or with the addition of partially purified proteins from fractionated cell extracts (4-7). Participating factors along with RNA polymerase II and the DNA template assemble in such a manner that initiation of transcription occurs *in vitro* at the same specific point on the DNA template as it occurs *in vivo* (2).

Studies using either deletion or point mutants with altered transcriptional efficiencies have shown that sequences of the DNA template apparently act as signals, directing transcriptional factors and RNA polymerase to the site of initiation (8-11). AT-rich signals located between -30 and -25 base pairs (bp) from the transcription initiation site (TATA box) have been defined as essential both for *in vivo* and *in vitro* transcription by deletion analysis (12).

It is well established that DNA sequences in the Goldberg-Hogness box (13) play some role in regulating mRNA synthesis. Other reports indicate that DNA

sequences surrounding and including known transcriptional initiation sites possess some regulatory function. Transcriptional activity was restored when DNA sequences from an active α -globin gene containing the initiation site, but not the TATA box (from -9 to +7), replaced those of an α -globin pseudogene (14). Also, deletion of DNA sequences of the adenovirus 2 (Ad2) major late transcription unit, which included the initiation site, quantitatively reduced transcriptional activity of that gene *in vitro* (15). In an effort to define the DNA sequences that act as signals for regulating the rates of mRNA synthesis, we constructed point mutants containing specific base changes in the Ad2 major late promoter that included the TATA box and the site of transcriptional initiation. Such single base changes in the TATA box (TATC: AC-28) are known to significantly reduce transcriptional efficiency compared to that with unaltered templates (16). We show here that transcriptional efficiency is reduced when the initiating base (+1) is changed (A→T: AT+1), so that all transcripts are initiated with pyrimidines. Furthermore, specific transcription is abolished when DNA templates contain both mutations (AC-28 and AT+1). These results suggest that the TATA box and the initiation site are involved as signals for molecules participating in mRNA synthesis, and indicate that both sites are essential for specific RNA polymerase II transcription.

MATERIALS AND METHODS

Construction of M13 Recombinants

The construction and characterization of M13/MLP recombinants has been described (16).

Synthesis of Oligodeoxyribonucleotides

The 20-nucleotide (n) oligodeoxyribonucleotide used in site-specific mutagenesis of the Ad2 major late promoter initiation site was manually synthesized on solid-phase supports by the phosphite procedure (17). The 21-n synthetic primer used in primer extension studies and the 15-n sequencing primer was synthesized using an Applied Biosystem 380A DNA synthesizer, and purified by high-performance liquid chromatography on a C-18 reverse-phase column. The purity of the oligonucleotides was verified by gel analysis of end-labeled products.

Site-Directed Mutagenesis

The procedure used for transferring point mutations from a synthetic oligonucleotide into M13 MLP DNA has been described (16).

Phage Plaque Hybridization Using Radiolabeled Synthetic DNA Probes

Radiolabeling of synthetic DNA probes and methodology of mutant M13 phage

selection were as before (16). Hybridized filters were washed in 6X SSC at increasing temperatures until differences in thermal denaturation between mutated and parental DNA sequences were observed.

DNA Sequence Analysis

Mutagenized DNA sequences were confirmed by the dideoxy-terminator method of Sanger *et al.* (18), using the single-stranded phage DNA as template and a 15-n single-stranded DNA primer.

In Vitro Transcription Assay

Extracts from HeLa cells (WCE), used for *in vitro* transcription, were prepared by the method of Manley *et al.* (2). Assay and processing conditions were as described (15). Transcription reactions were terminated after 60-min incubations at 30°C. DNA templates containing Ad2 and ϵ -globin genes were prepared by digestion with Bam HI and Nar I restriction enzymes, according to the conditions recommended by the supplier (New England Biolabs). The digestion was sequential because Nar I only cut efficiently linear DNA.

Primer Extension Assay

The 797-n RNA runoff transcripts from parental, AT+1, and AC-28 DNA templates were eluted from polyacrylamide gel slices by the method of Smith (19). Extension of cDNA from the 21-n synthetic DNA primer following hybridization to the specific runoff RNA or with RNA isolated from HeLa cells 18 hr after infection with Ad2 was performed by the method of Agarwal *et al.* (20). Briefly, 0.5 μ g of unlabeled cDNA primer and fmole quantities of RNA were heated in 5 mM sodium phosphate, pH 6.8, 0.15 M sodium chloride, and 5 mM EDTA (final volume, 5 μ l) to 90°C and allowed to cool slowly to 37°C. The mixture was then adjusted to 50 mM Tris-HCl, pH 8.0, 5 mM dithiothreitol, 7.5 mM MgCl₂ and 0.15 M NaCl, 40 μ Ci [α -³²P]dATP (> 7000 Ci/mM), and 10 μ Ci each of the remaining three α -[³²P]-labeled deoxyribonucleotide triphosphates (~3000 Ci/mM; Amersham Corp.). Reverse transcriptase (15 units) was added, and samples were incubated at 37°C for 45 min, followed by an additional 15 min after addition of all four unlabeled dNTPs to 200 μ M. After incubation, the reaction was terminated by addition of EDTA to 10 mM. Two volumes of ethanol/Na acetate were then added to precipitate nucleic acids. The pellets were washed with 80% ethanol, dried and dissolved in 3 μ l of 0.1 N NaOH, 2 mM EDTA, and incubated at 37°C for 30 min. Following this treatment, 7.0 μ l of formamide/bromophenol blue/xylene cyanol was added, samples were boiled for 3 min and electrophoresed through 20% polyacrylamide-7 M urea gels. Autoradiograms were obtained by exposing gels to Fuji RX film at -70°C.

Fig. 1. Schematic diagram of the parental recombinant DNA used in this study. Shown are the sites of cleavage by the respective restriction endonucleases that are the termination points of transcripts synthesized in vitro. Also shown are the DNA sequences inclusive of the Ad2 major late TATA box and site of transcription initiation as well as those of the synthetic 21-n cDNA used for primer extension analysis.

RESULTS

A schematic of the M13 parental recombinant DNA, mp9-MLP ϵ G, used for this study is shown in Figure 1. A 660 bp ϵ -globin DNA fragment was inserted into the Bam HI restriction site of the M13 and mp9-MLP recombinant that could be used as a control gene during the transcriptional analysis subsequent to mutagenesis.

We synthesized a 20-n oligonucleotide where the adenine at position +1 was substituted with a thymidine as described before (17). The mutagenesis procedure involves hybridization of the oligonucleotide to wild-type MLP single-stranded DNA, conversion to the double-stranded replicative form with the Klenow fragment of DNA polymerase I, repair of the gap with T4 phage ligase, transfection of the JM 103 bacteria, and plating. The mutagenized phages are selected by screening nitrocellulose lifts containing phage DNA using the end-labeled oligonucleotide and differential washing of the wild-type versus the mutant plaques. After a second screen, the change was confirmed by direct sequencing of the phage DNA. According to our convention (16), we called this mutant AT+1.

To determine the effect of this change on transcription, both parental and mutant DNAs were digested with Bam HI and Nar I restriction enzymes (see Fig.

Fig. 2. Autoradiogram of *in vitro* transcripts derived from DNA templates. The templates shown diagrammatically in Fig. 1 were digested with Bam H1 and NaeI restriction enzymes and incubated with HeLa whole cell extracts. The $\alpha[^{32}\text{P}]\text{GTP}$ -labeled RNAs were analyzed on 4% polyacrylamide-7 M urea gels run at 35 mA for 3 hr. In the autoradiogram, the RNA runoffs of the adenovirus major late promoter (797n) and the ϵ -globin (460n) genes are indicated by the arrows. Lane 1 contains RNA transcribed from the unmodified (wild type, WT) adenovirus major late promoter, lane 2 from the AT+1 mutant, lane 3 from the AC-28+AT+1 double mutant and lane 4 from the AC-28 mutant template. In the lower panel, a scan of the autoradiogram depicts the AC-28 mutant (full line) and the double mutant (dashed line). The size of the scan was adjusted so that the two major peaks coincide with the bands that correspond to the plasmid and ϵ -globin (460n) transcripts.

1). The resulting DNA fragments were incubated with a cell extract prepared from HeLa cells as described by Manley *et al.* (2). *In vitro* transcription of wild-type DNA templates results in two specific runoff RNA transcripts of ϵ -globin (460 n) and MLP (797 n) (Fig. 2, lane 1) as predicted. DNA templates containing the AT+1 substitution in MLP resulted in reduced levels of the 797-n adenovirus runoff transcripts (Fig. 2, lane 2). Approximately 50% of the radioactivity ($[\alpha\text{-}^{32}\text{P}]\text{GTP}$) was incorporated into the specific RNA product derived from AT+1 templates when compared to those from unaltered templates, as determined by direct liquid scintillation counting of the gel slices in eight separate experiments. While these differences were observed between mutant and wild-type transcripts, no such differences were observed in ϵ -globin-derived transcripts that were synthesized from templates present in concentrations exactly equimolar to the MLP templates. Thus we conclude that the site of ini-

tiation is partially responsible for regulating transcription efficiency, by fulfilling some sequence requirement.

To gain some insight into the relationship between the TATA box and initiation site, a double mutant was constructed. The 20-n long oligonucleotide was used to mutagenize our previously described AC-28 TATA box mutant. The resulting double mutant, confirmed by direct sequencing, contained two base changes, one within the TATA box and one at the +1 position. These templates were digested with Bam HI and Nar I and the effect of both base changes on transcription was assessed. As previously reported, introduction of base changes within the TATA sequences reduces levels of transcription in vitro to different extents, depending on the position of change (16). As shown in Figure 2 (lane 4), transcription of AC-28 ϵ G DNA results in reduced levels of mutant MLP runoff transcripts relative to those from the ϵ -globin internal control, wild-type MLP and AT+1 DNAs. However, no transcripts were detectable when the double mutant AC-28•AT+1 DNA templates were incubated with the whole cell extract (Fig. 2, lane 3). A scan of the autoradiogram for lanes 3 (dotted line) and 4 (full line) is shown under the autoradiogram. No detectable peak can be detected in the AC-28•AT+1 double mutant. Specific runoff transcripts were undetectable from these templates on other autoradiograms of longer exposures or when only 1/10 the amount of the AC-28 mutant template product was analyzed on the gel and detected by autoradiography. In this particular experiment (Fig. 2, lane 4) the AC-28 transcription level was less than the 20% of wild-type value previously reported (16). If the effect of the AT+1 and the AC-28 mutations were additive, we still would have easily detected transcription, despite the combined reduction. Other experiments also failed to detect any transcripts from the double mutant when autoradiograms were grossly overexposed (not shown), or when transcription levels were assayed at various DNA concentrations (not shown). In all cases where the AC-28 or AC-28•AT+1 MLP mutants were transcribed simultaneously with the ϵ -globin internal standard, higher levels of the latter were detected. Moreover, the AC-28 and AC-28•AT+1 mutants are unable to compete against wild-type MLP DNA in a regular transcription assay (our unpublished observations).

Primer Extension Analysis of In Vitro Transcripts

As shown above, base changes at positions -28 and +1 reduce transcription efficiency. To determine the effect, if any, of these mutations on the accuracy of transcription initiation, we examined the 5' ends of the in vitro transcripts, using primer extension analysis on specific RNAs synthesized from wild-type DNA templates and those from templates with single base changes. cDNA

was extended with labeled dNTPs from the 3' end of a 21-n synthetic primer that was base-paired to the +4 position of the RNA (see Fig. 1) when hybridized to specific 797-n RNA excised from polyacrylamide-urea gels. The amount of specific transcripts synthesized *in vitro* using HeLa extracts is usually on the order of fmoles. To compensate for the reduced transcription of AT+1 and AC-28, proportionally larger amounts of RNA runoffs were used for these mutants. To enhance the sensitivity of this analysis, all four ^{32}P -labeled dNTPs were added to primer extension reactions. Furthermore, one is assured that bands observed on autoradiograms result from primer extension rather than from any remaining labeled RNA since the specific activities of the cDNA are 10^2 - 10^3 times greater than those of the labeled RNA. Since the RNAs analyzed were selected as discrete bands from polyacrylamide gels, great differences in the 5' ends of these molecules were not expected. Rather, it was of interest to determine whether 5' end heterogeneity of one or more bases could be detected in RNAs resulting from mutation of the DNA templates at the transcription start sites. To insure detection of the possible minor differences, it was more useful to employ a primer placed near the expected 5' end so that when extended, the difference between single base length products could be easily resolved on polyacrylamide gels. As a control for potential artifacts arising from cDNA synthesis using reverse transcriptase, RNA isolated from cultured HeLa cells 18 hr after Ad2 infection was included in the analysis.

The results of the primer extension analysis are shown in Figure 3. As expected, the identical 5' ends of transcripts synthesized from wild-type templates *in vivo* (lane 1) and *in vitro* (lane 2) corresponded predominantly to the +1 position, with relatively small amounts of RNA from +2 and -1 positions. This was not the case for *in vitro* synthesized RNA from AT+1 (lane 3) and AC-28 (lane 4) templates. The 5' ends appeared to be more heterogeneous with a greater proportion starting at the -1 and +2 positions, especially in the case of the AT+1 mutant DNA, as compared with RNA synthesized from wild-type templates. Since this primer extension analysis starting from position +4 (Fig. 1) depends on the addition of radiolabeled dNTPs, all four being present during reactions, quantitative interpretation of the frequency of initiation at different positions is complicated. However, since the longer products contain more radioactive nucleotides, the -1 starts in the AT+1 mutant (with four labeled deoxynucleotide triphosphates incorporated) are less frequent than the +2 (with two labeled deoxynucleotide triphosphates incorporated). Similarly, the very long extension products observed in AC-28 probably represent very rare RNA molecules. Extension products indicating transcription start sites at +3 were not detected.

Fig. 3. Primer extension analysis of transcripts synthesized *in vivo* and *in vitro*. A synthetic 21-n cDNA was hybridized to the 797n RNA runoff eluted from a gel like the one shown in Fig. 2 and subsequently incubated with 32 P-dNTPs and reverse transcriptase (see Materials and Methods). Primer extension products derived from: lane 1, cytoplasmic RNA from HeLa cells 18 hr after Ad2 infection; lane 2, *in vitro* transcripts from wild-type DNA template; lane 3, *in vitro* transcripts from AT+1 DNA template (RNA was from a reaction twice the size of the one in lane 1); lane 4, *in vitro* transcripts from AC-28 DNA template (RNA was from a reaction 10 times as large as that in lane 1); lane 5, 32 P-end-labeled 21-n cDNA.

DISCUSSION

Although *E. coli* RNA polymerase shows a strong preference for purines over pyrimidines, similar studies with specific RNA polymerase II transcription have not been very exhaustive. The 5' end of capped mRNAs, which accurately reflect the transcription initiation site (23, 24), usually contain a purine, although some pyrimidine 5' ends have been reported. Adenovirus transcripts from EIV, for example, are initiated with U or A at approximately equal frequencies (24). Recently, Tokunaga *et al.* (25) reported a 30% reduction of fibroin gene transcription as a result of a purine-purine (A+G) substitution at the start site. We have studied the effect of base changes within the Ad2 major late promoter TATA box sequences and at the transcription initiation site on *in vitro* transcription. Changing the initiating A to T resulted in reduced levels of accumulated specific transcripts. The mutation also resulted in an increased utilization of the +2 start site. Thus, *in vitro* synthesized RNAs are initiated either with a U (+1) or a C (+2) with transcription efficiencies which are half

of those in the wild-type. The only other purines available close by are Gs located either at +11 or at -6, although only the latter would be detected with our assay. This suggests that distance from the TATA box is a much stronger determinant of the initiation site than the purine or pyrimidine nature of the 5' nucleotide. A certain flexibility in the selection of the start site probably remains, and it will be interesting to investigate the effect of reintroducing purines at varying distances from the +1 position into the AT+1 mutant to systematically assess this flexibility. The AT+1 mutant will also be useful in the analysis of the β - γ -ATP hydrolysis requirement (23), since the first A occurs in the sequence at position +13.

As shown previously (16) and in this report, transcription was quantitatively reduced as a result of a base change at position -28, which occurs within the adenovirus TATA sequences. Neither the base change at -28 nor that at +1 alone were sufficient to abolish transcription, although the resulting reduction in the levels of accumulated transcripts indicates their involvement in regulating this process. In addition, changes either in the TATA box or at the initiation site resulted in increased heterogeneity of the transcription start sites. The TATA box seems to function both in vivo and in vitro as a reference point such that initiation of transcription occurs 30 nucleotides downstream. The nucleotide sequences around the initiation site itself appear to be recognized, at least partially, by the element involved in RNA polymerization, since RNAs are synthesized at different efficiencies when these sequences are altered. Thus, there is some relationship between the site of reference (TATA box) and the initiation site in normal transcription. In the double mutants, the constraint imposed by the nucleotide changes eliminates transcription below the limits of detectability. The effect of both base changes together was not additive since expected levels of approximately 1% that of the wild-type would have been easily detected using the runoff assay for transcription. Rather, the double mutant effect observed indicates some coordinated process involving both sites. Perhaps one or more protein factors bind at these sites on the DNA molecules and act in some "docking" function for the RNA polymerase, thereby conferring sequence specificity. Those factors reportedly responsible for commitment to specific transcription (21) may be involved in such a process. This idea is consistent with our results from primer extension analysis of 5' ends of in vitro transcripts, whereby mutations at either one of the positions reported here would probably destabilize transcription complex formation sufficiently to affect both transcription efficiency and accuracy of initiation. The inability of these mutants to compete with either the ϵ -globin

DNA or the Ad2 MLP DNA (results not shown) suggests that the transcriptional factors interacting with this region of the DNA are either not bound or inefficiently bound to the mutant promoters. Several lines of evidence available implicate regions surrounding the transcription initiation site in determining the efficiency of a RNA polymerase II-specific promoter: i) deletion-substitution mutants which include the initiation site show reduced levels of transcription (9, 15, 25, 26); ii) point mutants in this region of the ovalbumin (5), globin (14, 26), silk fibroin (25), and, as shown in this study, the adenovirus major late promoter show an effect on the rate of promoter utilization, although analysis of linker scanning-mutants revealed no effect in a heterologous transcription system (27); iii) in *Drosophila*, a region spanning nucleotides -44 to +12 interacts with one or more partially purified transcription factors as detected by footprint analysis (22); and iv) DNA templates truncated 33 nucleotides downstream of the initiation site (+33) cannot be utilized in vitro (28, 29, 30). This evidence strongly supports the notion that transcription factors interact both with the region around the initiation site and directly or indirectly with the TATA box region.

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