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Protein factor(s) binding independently to two different regions of the adenovirus 2 major late promoter

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ABSTRACT

The protein factor(s) in a fraction from the HeLa cell nuclear extract required for specific *in vitro* transcription can specifically bind to adenovirus 2 major late promoter (Ad 2 MLP) DNA. We demonstrate by *in vitro* footprinting assay that there are two asymmetric protected regions covering the TATA box and the nucleotides upstream from the TATA box. In the coding strand, the DNAse I protected regions span from nucleotides -10 to -50 and from -52 to -68. In the noncoding strand, the protected regions span from nucleotides -10 to -32 and from -45 to -65. Using different Ad 2 MLP point mutants in this assay, we show that the transcriptional down mutants of the TATA box (AC-30 and AC-28) abolish the binding of protein factor(s) to the TATA box but do not affect binding in the upstream region. The new upstream transcriptional down mutant (TA-56) abolishes the binding of protein factor(s) in the upstream region but does not affect binding to the TATA box. The mutants which do not affect transcription efficiency (GA-51 and CG-61) do not modify the binding to either the TATA box or the upstream region. Methylation protection experiments show that the guanosines at -58 and -60 in the coding strand and at -57 (probably also -55) in the noncoding strand are in close contact with protein factor(s). The results indicate that the TATA box and its upstream region of Ad 2 MLP are independently bound by at least two different factors *in vitro*.

INTRODUCTION

In the eukaryotic RNA polymerase II transcription process, multiple sequences upstream from the start site are defined as essential for efficient function. They include several promoter elements such as the TATA box and upstream regions, enhancers, some transacting response sequences and some repressor sequences. Some of these sequences might also be located downstream from the transcription start site. Most of these sequences appear to exert their effect on transcription by binding of different protein factors (1-9).

The adenovirus 2 major late promoter (Ad 2 MLP) is responsible for the transcriptional control of mRNAs encoding a series of early and late viral proteins (10). This promoter functions very efficiently *in vitro* in a cell-free system (11) and has been studied extensively. Deletion and point mutations on Ad 2 MLP indicate that the TATA box and cap site are both necessary

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for the specific initiation of transcription (12-14). The third element, a sequence upstream from the TATA box up to nucleotide -97, is also considered essential for efficient transcription (6, 15) and will be designated UPE (upstream promoter element).

We have previously constructed two point mutants of the TATA box on Ad 2 MLP, i.e., AC-30 and AC-28, and have reported that their transcriptional activities *in vitro* using HeLa whole cell extracts are reduced by 50 to 80% (12). A new point mutant located upstream from TATA box, TA-56, can decrease the transcription efficiency by nearly 50%. These Ad 2 MLP DNA point mutant variants seem to be ideal tools for studying DNA-protein interactions, which can be compared directly with their transcription abilities.

By using different deletion templates in an indirect transcription competition assay, Davison *et al*. (3) showed that the TATA box region is involved in "stable" binding of a transcription factor. Miyamoto *et al*. (6) reported that the region upstream from TATA box from nucleotides -34 to -97 also involves the binding of a different specific factor(s) because the TATA box sequences could not compete the binding function of the upstream region in transcription. We report here on the footprint of proteins that are contained in a nuclear extract fraction required for specific transcription and that interact with specific regions of the Ad 2 MLP DNA. This DNA region corresponds well with the interactions detected previously *in vivo* and in a whole cell extract (Shi, X. P. and R. Weinmann, submitted). Point mutants of the Ad 2 MLP promoter with reduced transcriptional activities show altered footprint patterns that are compatible with at least two independent DNA binding sites, one at the TATA box and one located upstream (UPE).

MATERIALS AND METHODS

Construction of Ad 2 MLP Mutants

The construction and characterization of the M13/MLP recombinants, the synthesis of oligodeoxyribonucleotides and the site-directed mutagenesis procedures as well as selection of mutant M13 phages have been described (12,13). The mutagenized DNA sequences were confirmed by the dideoxy chain termination method (17).

The *in vitro* transcription efficiency of the Ad 2 MLP mutants was measured in a run-off assay using a whole cell extract and compared to an ε-globin gene internal standard contained on the same DNA molecule (12,13).

Preparation of Fraction C

A HeLa cell nuclear extract was prepared and fractionated according to

Dignam et al. (18). The nuclear extract was fractionated on a phosphocellulose (Whatmann P11) column and eluted in a step gradient of 0.1, 0.3, 0.5 and 1.0 M KCl containing a buffer of 20 mM Hepes, pH 7.9, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.5 phenylmethylsulfonyl fluoride (PMSF) and 20% glycerol. The activity of each fraction was checked by reconstituting in vitro transcription reactions. The yield of 536n runoff was as good as with the unfractionated extract and required 1 µl of the 0.1 M step, 4 µl of the 0.5 M step (fraction C) and 4 µl of the 1 M KCl step in a 20-µl reaction.

Footprint Analysis

The Ad 2 MLP replicative form [RF] DNA (13) was cut by restriction enzyme HindIII (for the noncoding strand) or by AvaII (for the coding strand). The cut ends were filled and labeled with the Klenow fragment of DNA polymerase I (Bethesda Research Labs) in the presence of all four α-[^{32}P]deoxyribonucleotide triphosphates (3000 Ci/mM, Amersham). The DNA was cut again with restriction enzyme AvaII (for the noncoding strand) or with DdeI (for the coding strand). The labeled DNA fragments were separated on a non-denaturing polyacrylamide gel, cut out and eluted from the gel.

Footprint assays were carried out under the same conditions as the in vitro transcription assay using unlabeled pBR322 DNA as carrier. Approximately 3 to 5 x 10^4 cpm of labeled DNA was used for each assay. The final concentrations in the reactions were $MgCl_2$ (7.5 mM); KCl (50 mM), glycerol (10%), Hepes, pH 7.9, (10 mM) and DTT (0.5 mM). Ten µl of fraction C was added to the 20-µl reaction mixture, and incubated at room temperature for 5 to 10 min. Fresh diluted DNAse I (Sigma) was added for 1 to 3 min and the reaction was stopped by adding 1 volume of 20 mM EDTA, 20 mM Tris/HCl, pH 8.0, 0.2% sodium dodecyl sulfate (SDS) and 50 µg/ml tRNA. After phenol extraction, the DNA was precipitated, denatured and analyzed on a polyacrylamide/urea gel.

For dimethylsulphage (DMS) cleavage footprint analysis the same DNA fragments used for DNase I footprint analysis were incubated with fraction C for 5 min under the in vitro transcription conditions described above. DMS (1 µl; Aldrich Chemical Company) was added for 0.5 to 1 min at room temperature. The reaction was terminated and samples were treated as described by Maxam and Gilbert (19) for sequencing.

RESULTS

Interactions between proteins contained in the nuclear extract and Ad 2 MLP DNA were studied using the footprinting technique of Galas and Schmitz (20). Two DNA fragments of Ad 2 MLP, an AvaII-HindIII fragment and an

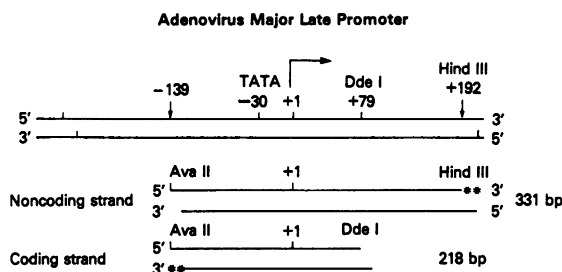

Fig. 1. Schematic representation of the Ad 2 MLP and the DNA fragments used for in vitro footprinting assay. The arrow above the lines indicates the start site and direction of transcription from the Ad 2 MLP; the numbers were given in relation to the transcription start site (+1). The TATA box and the cleavage sites of several restriction enzymes used in our experiments are indicated. For showing the protection in the noncoding strand, the AvaII-HindIII restriction enzyme 331-base pair (bp) DNA fragment was used, which was labeled at the 3' end at the HindIII cleavage site; for the coding strand, the AvaII-DdeI restriction enzyme 218-bp DNA fragment with the 3' end labeling at the AvaII site. The labeling site is indicated by an asterisk.

AvaII-DdeI fragment, were labeled in the noncoding (l) and coding (r) strands, respectively (Fig. 1). Because nucleases contaminating nuclear or whole cell extracts prevent clear footprinting with end-labeled DNA fragments, the HeLa nuclear extract was fractionated through a phosphocellulose II column. Four fractions, A, B, C, D, were obtained after elution with 0.1 M, 0.3 M, 0.5 M, and 1.0 M KCl, respectively. Only fractions A, C, and D were necessary to reconstitute in vitro transcription from the Ad 2 MLP gene, as demonstrated by others (18).

Fractions A, C, or D were incubated individually with end-labeled fragment under transcription conditions and treated with DNAse. The footprinting assay showed that most of the specific Ad 2 MLP DNA binding activities are contained in fractions C and D. Results are given for experiments using fraction C which contains approximately 7 to 10% of the total protein in the HeLa nuclear extract. This impure fraction still contains some of all required transcriptional factors contained in fractions A, B and D, since at sufficiently high concentrations, runoff transcripts can be detected. (D. Reinberg, personal communication).

The effect of DNA concentration on the footprint analysis with fraction C was determined by adding unlabeled pBR322 to a constant amount of end-labeled Ad 2 MLP DNA fragment. The results indicate that increasing amounts of pBR322 can effect an overall reduction in protection and increase the specificity of

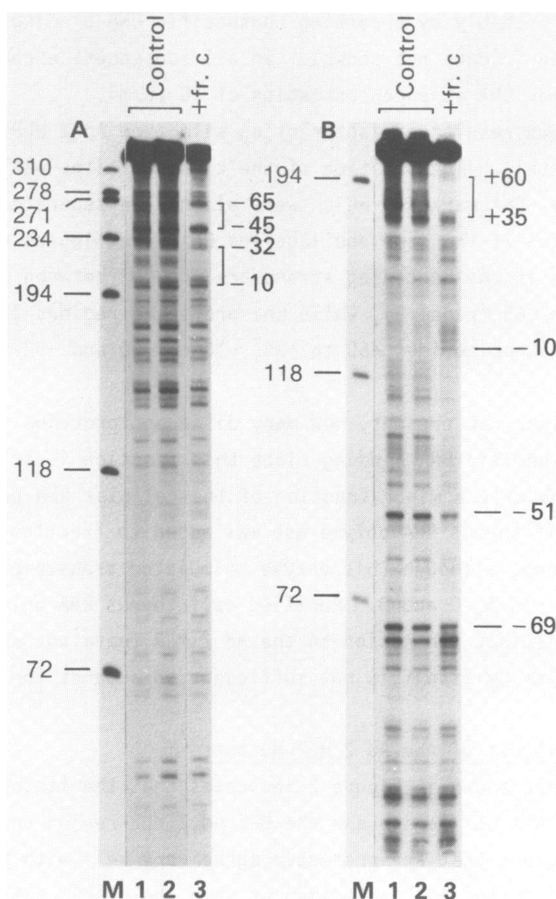

Fig. 2. Footprint analysis of the fraction C on Ad 2 MLP in vitro. Panel A shows the footprint analysis on the noncoding strand and panel B on the coding strand. All samples contained 10 µg/ml pBR322. Lanes 1 and 2 are naked DNA cleaved by DNAse I (end concentration, 1 µg/ml) for 30 seconds (lane 1) and 1 min (lane 2) at room temperature. Lane 3 shows the results in the presence of fraction C, which was eluted with 0.3 to 0.5 M KCl from a phosphocellulose column loaded with HeLa nuclear extract. The fraction (10 µl per reaction) was incubated with pBR322 carrier and end-labeled DNA fragment at room temperature for 10 min. The DNAse I was added to 1 µg/ml and incubation continued for 2 min. The reaction was stopped by adding EDTA and SDS (see Materials and Methods). DNA was purified and analyzed on a polyacrylamide/urea gel. The numbers on the right side as well as the determination of the size of the analyzed DNA fragments are explained in the Methods section. M in Panels A and B indicates the size marker of HaeIII-digested end labeled φx 174 DNA, which were aligned in separated experiments with a sequencing reaction of the same end labeled DNA fragment (see Figures 6 and 7).

protein binding, probably by absorbing nonspecific DNA binding proteins contained in fraction C (data not shown). In all subsequent experiments, pBR322 was used as carrier DNA at a concentration of 10 μg/ml.

Figure 2 shows results of footprinting wild-type Ad 2 MLP DNA with fraction C in more detail. The position of the cleavage sites was determined from the size of the ϕx DNA markers, which were aligned in separate experiments with a sequencing reaction of the same end labelled DNA (see figures 6 and 7). The protected regions in the noncoding strand are located between nucleotides -10 to -32 and -45 to -65 (panel A), while the protected regions in the coding strand are between nucleotides +60 to +35, -10 to -50 and -52 to -69 (panel B).

It is not clear, at present, how many different proteins in fraction C are involved in specific DNA binding since this fraction is still relatively crude and contains only a minor fraction of the cellular RNA polymerase II. When purified calf thymus RNA polymerase was added to fraction II C, the footprint was unaltered, although this enzyme stimulated transcription when combined with fractions A, C and D. Purified calf thymus RNA polymerase II did not reveal any distinct protection on the Ad 2 MLP (data not shown), suggesting that the enzyme by itself is not sufficient to account for the footprint pattern.

Transcription Analysis of the Ad 2 MLP Mutants

The experiment shown in Figure 2 indicates that the factors in fraction C can bind to the TATA box region and the UPE Ad 2 MLP region on both DNA strands. The regions that are protected agree very well with those interacting with proteins during Ad 2 infection or when whole cell extracts were incubated with Ad 2 MLP DNA (Shi and Weinmann, submitted). To further establish the significance of these sequences in transcription, we analyzed five point mutants in these DNA regions. The results of a typical *in vitro* transcription reaction with these mutant templates are shown in Figure 3. Two run offs, the 850n MLP RNA and the internal control 460n ε-globin RNA are shown at different template concentrations. AC-28 and AC-30 are TATA box mutants with a reduction of transcription efficiency of approximately 80% and 50%, respectively) (13); TA-56 is a new UPE mutant with transcription reduced to 50%; GA-51 and CG-61 are mutants with no significant reduction in transcription efficiency. The levels of transcription are averages of 3 different experiments, normalized with the ε-globin standard and calculated at the optimum DNA concentration. A summary of the phenotypes of these mutants is presented in Figure 8. A more detailed analysis of their effect on transcription both *in vivo* and *in*

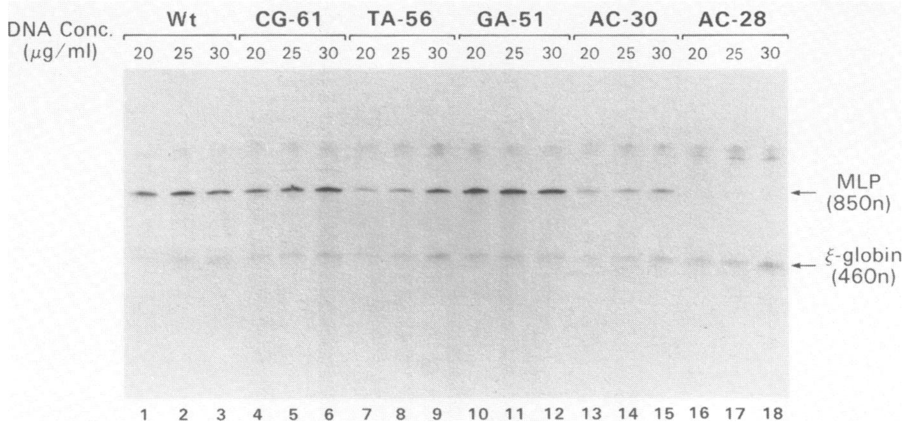

Fig. 3. In vitro transcription assay of the Ad2 MLP mutants.
Ad2 MLP mutant DNAs from the Xho to HindIII fragment and a Bam H-1 ε-globin fragment were inserted into a mp18 vector. The ε-globin served as an internal control. The plasmids were digested with BamH1 and BglII restriction enzymes and incubated with HeLa whole cell extracts. The α-^{32}P GTP-labeled RNA run-off products were analyzed on 4% polyacrylamide-7M urea gels (13) and are indicated by the arrows in the autoradiogram. Lanes 1 to 3 show RNA run-off from wild type Ad2 MLP, lanes 4 to 6 from the mutant CG-61, lanes 7 to 9 from the mutant TA-56, lanes 10 to 12 from the mutant GA-51, lanes 13 to 15 from the mutant AC-30 and lanes 16 to 18 from the mutant AC-28. The DNA concentration is indicated on the top of each lane. For quantitation the bands were excised and counted in a liquid scintillation counter with Aquasol-2 fluid (New England Nuclear).

vitro, will be presented elsewhere (Lee, Concino and Weinmann, in preparation).

Footprint Analysis of the Ad 2 MLP Mutants

Figure 4 shows the fraction C footprint analysis on those five mutants in the noncoding strand. The control DNase I experiments using naked DNA for each one of the mutant DNAs did not show any differences with wild type (data not shown). In the wild-type (Figure 2, lanes 1 and 2), there are two protected regions, -10 to -32 and -45 to -65. In AC-28 mutant there is no clear protection at the TATA box region (-10 to -32), but the UPE region (-45 to -65) remains protected (lane 3). The AC-30 mutant has a hypersensitive site at position -30 (lane 4). Since the control sample with the same mutant DNA in the absence of fraction C does not show this hypersensitive site (lane 8), it must result from a different interaction between the mutant DNA and protein factor(s) contained in fraction C. Like AC-28, the AC-30 mutant also does not influence the binding of the UPE sequences in the same DNA. The protection of mutants GA-51 and CG-61 is indistinguishable from wild-type, in good correlation with the results of in vitro transcription efficiency (lanes 5 and 7,

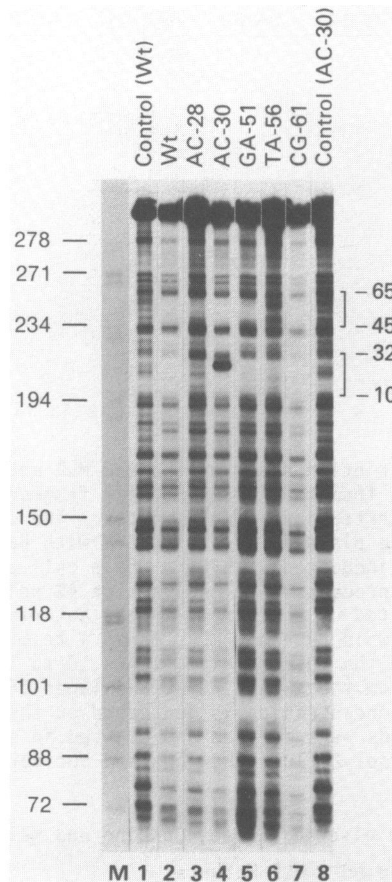

Fig. 4. Footprint analysis of the Ad 2 MLP mutants on the noncoding strand. All reactions contained 10 µg/ml pBR322. Lane 1 shows the naked DNA control with wild-type Ad 2 MLP, and lane 8, the naked DNA with the AC-30 mutant. Reactions shown in lanes 2 to 7 were all carried out in the presence of 50% of fraction C (see legend of Figure 3) with the wild-type Ad 2 MLP (lane 2), the AC-28 TATA box down mutant (lane 3), the AC-30 TATA box down mutant (lane 4), the GA-51 mutant (lane 5), the TA-56 down mutant (lane 6), and the CG-61 mutant (lane 7). The conditions were the same as described in the legend to Figure 3. The left lane M shows the end labeled, HaeIII and HhaI digested ϕx174 DNA size marker.

respectively). In contrast, in the TA-56 down mutant, only the TATA box is protected and not the UPE region (-45 to -65, lane 6).

The results from the coding strand of the same mutants are shown in Figure 5. Although the difference in footprinting patterns between down mutant AC-30 and wild-type Ad 2 MLP is not as pronounced as with the non-

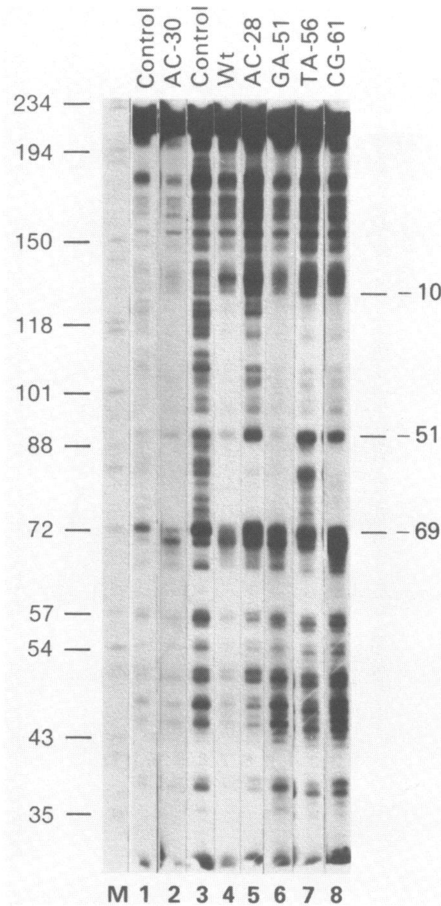

Fig. 5. Footprint analysis of the Ad 2 MLP mutants on the coding strand. All reactions were carried out under the same conditions described in the legend of Fig. 3. Lanes 1 and 3 show the naked DNA control with light and heavy exposures, respectively, and lane 2 shows the mutant AC-30. Lanes 2 and 4 to 7 show the reactions in the presence of 50% fraction C with the wild-type Ad 2 MLP (lane 4), the AC-28 TATA box down mutant (lane 5), the GA-51 mutant (lane 6), the TA-56 down mutant (lane 7), and the CG-61 mutant (lane 8). The left lane M shows the same size marker as described in the legend of Figure 4.

coding strand, the two other down mutants, AC-28 and TA-56, do show significant differences in their protection pattern. The TATA box region (from -10 to -51) of AC-28 (lane 5) and the UPE region (between -51 and -69) of TA-56 (lane 7) are not as well protected as either the wild-type or as the mutants with unaltered transcription efficiency.

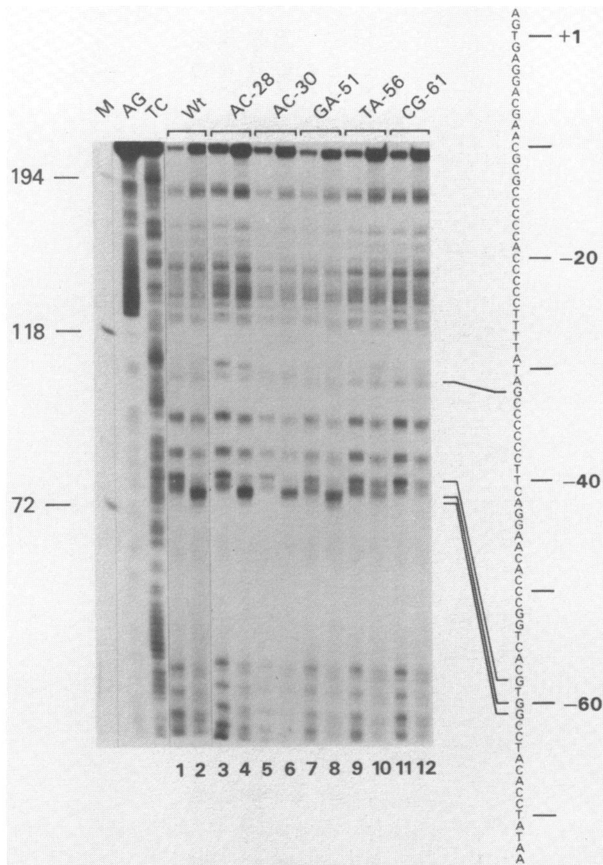

Fig. 6. Methylation protection experiments with Ad 2 MLP mutants on the coding strand.

The same end-labeled DNA fragments used in Fig. 5 were incubated with fraction C described in Fig. 2 under in vitro transcription conditions. DMS was added to 5% for 0.5 to 1 min at room temperature. The sequencing reactions were carried out as described by Maxam and Gilbert (19). The Ad2 MLP mutants were analyzed in the absence (lanes 1, 3, 5, 7, 9 and 11) and the presence of fraction C (lanes 2, 4, 6, 8, 10 and 12). On the right side of the Fig. shows the + 1 to - 70 coding strand sequences from Ad2 MLP and the alignment at relevant positions. On the left side of the Fig. we show the size marker of HaeIII digested, end-labeled ϕx 174 DNA and sequencing purine or pyrimidine reactions with the same end labeled DNA fragments, as indicated.

Dimethylsulfate (DMS) Footprints

To further map the close DNA-protein contacts in the protected regions of Ad 2 mutants, we used DMS to cleave unprotected guanines in the DNA chain. The end labeled DNA fragments from the Ad2 MLP mutants were incubated in the

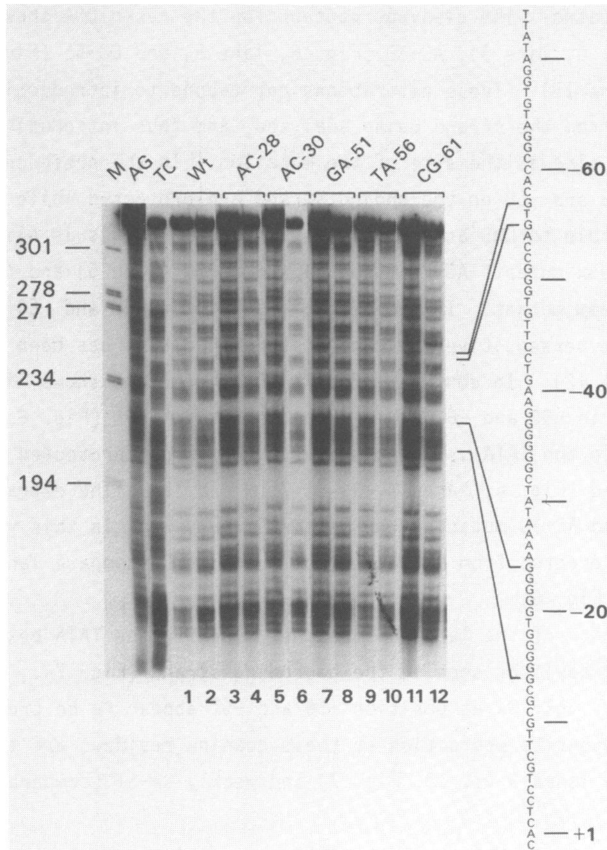

Fig. 7. Methylation protection experiments with Ad2 MLP mutants on the noncoding strand.

The same AvaII-Hind III fragments as in Fig. 4 were used. The reaction conditions were described in Fig. 6. The wild type (lanes 1 and 2) and the point mutants (lanes 3 to 12) of Ad2 MLP were analyzed in the absence (lanes 1, 3, 5, 7, 9 and 11) and presence of fraction C (lanes 2, 4, 6, 8, 10 and 12). On the right side of the figure shows the -70 to +1 noncoding strand sequences from Ad2 MLP with the alignment at relevant positions. On the left side the size marker of HaeIII-digested, end-labeled ϕx 174 DNA and the purine/pyrimidine sequencing reactions.

absence (odd numbered lanes, Figs. 6 and 7) or in the presence of fraction C (even numbered lanes, Fig. 6 and 7). The samples were treated with DMS and processed as described in Materials and Methods. Figure 6 shows the results obtained with the coding strand and Figure 7 the ones obtained with the noncoding strand. The alignement with size markers and regular sequencing reactions (three unnumbered lanes) with the Ad2 MLP sequence on the right of the

figure is indicated. The cleavage pattern for the naked DNA shows changes for the AC-28 (Fig. 6, lane 3), AC-30 (Fig. 6, lane 5, and CG-61 (Fig. 6, lane 11 and Fig. 7, lane 11). These alterations correspond to introduction or removal of G residues from the strand being analyzed, and thus introducing or removing a DMS cleavage site at the site of the mutation. In the presence of fraction C, the Gs at-58 and -60 on the coding strand are protected while the -G at -61 is very accessible to DMS attack (Figure 6, lane 1). This is also observed for both TATA box mutants AC-28 (lane 3) and AC-30 (lane 5) and the GA-51 (lane 7) upstream mutant. In the case of CG-61, the -58 and -60 Gs are protected but no hypersensitive -61 can be seen because it has been changed to a C (Fig. 6, lane 12). In contrast, the TA-56 down mutant shows little protection at the Gs in -58 and -60 and no hypersensitive G-61 (Fig. 6, lane 10). The G closest to the TATA is located at -32 and is not protected in any of the templates tested (Fig. 6, lanes 1, 3, 5, 7, 9 and 11). The extra Gs present in the AC-28 and AC-30 mutants (the latter is very weak in this region) do not appear to be protected from DMS attack by fraction C (compare lanes 3 vs. 4 and 5 vs. 6 in Fig. 6).

No protection of the large G stretches flanking the TATA box (-14 to -24 and -33 to -38) could be seen in the noncoding strand (Fig. 7). In the upstream region, only Gs at position -55 and -57 appear to be protected. Possible exceptions to protection at these guanine residues are the TA-56 mutant (compare lanes 9 vs. 10, Fig. 7) and weakly GA-51 (compare lanes 7 vs. 8, Fig. 7).

In summary, those mutants that affect transcription seem to affect the binding of specific DNA regions, but the binding to the TATA box does not seem to be affected in mutants in the UPE region and viceversa.

DISCUSSION

In this report, we demonstrate by an *in vitro* footprinting assay that the protein factor(s) in a fraction from HeLa nuclear extract required for *in vitro* transcription can bind specifically on the Ad 2 MLP DNA. Our results provide direct evidence that both the TATA box and the UPE region are specifically recognized and bound by protein factors. Since the fraction partially purified from HeLa nuclear extract and used in footprinting assay, is indispensable in the *in vitro* reconstituted transcription reaction, it is likely that the proteins which specifically bind to the regions defined as essential for Ad 2 MLP function are involved in transcription. This fraction represents 7 to 8% of the total proteins in the nuclear extract and represents a 20-fold

purification over a whole cell extract. Since the transcriptional factors seem to copurify through initial chromatographic steps (4,22), the simultaneous interactions of several different proteins required for transcription might be responsible for the footprint observed. A more detailed analysis of the protein-DNA interactions will require the use of extensively purified transcription factors.

There are two protected regions, covering the TATA box and the UPE region, upstream from the TATA box. In the coding strand, the protected regions are located from -10 to -50 and from -52 to -68. In the noncoding strand, the protected regions are located from -10 to -32 and from -45 to -65. While the DNAse I protection experiments delineate rather large regions of protein-DNA interaction (22 to 40 nucleotides for the TATA box and 16 to 20 for the UPE), the close contacts detected by the use of DMS seem to be limited to a few nucleotides. This difference in the size of the protected regions is probably related to the size of the probes themselves. DNAse I is more hindered in its function by the bound transcriptional factors than the small DMS molecule.

Carrier DNA in the reaction reduces the nonspecific binding of proteins and therefore increases the specificity of the interaction between protein factors and the Ad 2 MLP DNA. The protected region in the coding strand, located at nucleotides +35 to +60, could be related to transcription factor X, required for efficient transcription of MLP templates longer than +70, but not needed for those shorter than +33 (D. Reinberg, M. Horikoshi and R. G. Roeder, submitted for publication).

The experiments with deletion and point mutants indicate that the TATA box region -32 to -12 is absolutely necessary for _in vitro_ transcription (22), while the cap site (13) and the UPE region between -51 and -66 (6,15,16) are very important for the transcription efficiency. _In vitro_ competition experiments have implied that the TATA box and its upstream promoter element (UPE) could be bound by different factors in the HeLa whole cell extract (3,6). The effects of previously described down mutants, AC-28, AC-30 (13), and a series of new upstream mutants on transcription efficiency are summarized in Figure 8. By using different point mutants in the DNase I footprinting assay, we show that the down mutants of the TATA box modify the binding of protein factor(s) on the TATA box but do not affect the binding on the UPE. The upstream TA-56 down mutant modifies the binding of protein factor(s) on the UPE but does not affect the binding on the TATA box. The mutants, which do not significantly reduce transcription efficiency, do not affect the binding

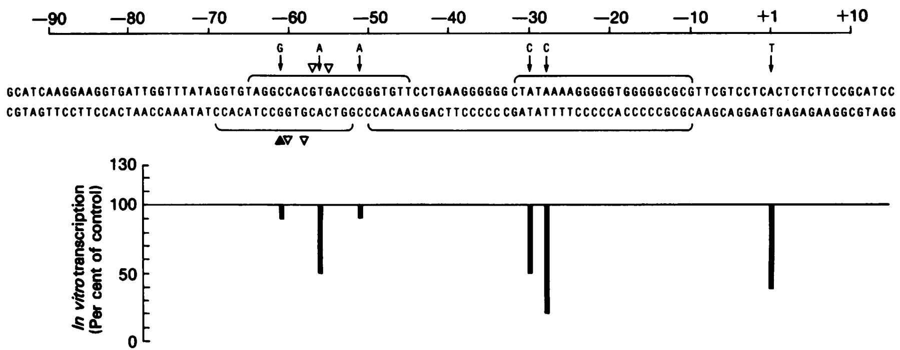

Fig. 8. Diagramatic summary of the protected regions in the footprint assay of Ad 2 MLP and the transcription efficiency of the Ad 2 MLP point mutants *in vitro*. The Ad 2 MLP DNA sequences from -90 to +10 are represented. The upper sequences correspond to the noncoding strand and the lower one, to the coding strand. The numbers indicate the number of base pairs away from the start site of transcription (+1). Sequences protected from DNAse I cleavage are shown by lines alongside the sequences of Ad 2 MLP. The triangles indicate the results from DMS protection experiments; the open triangles indicate the Gs protected from DMS and the solid triangle indicates the G hypersensitive to DMS. The nucleotide changes in different mutants can be located by the arrow which indicates the mutation site and the respective base substitution.

The lower part of the diagram represents the transcription efficiencies of the different Ad 2 MLP mutants relative to an internal ε-globin control, as described before (12,13). One hundred percent represents the level approached by wild-type, as shown in Fig. 3. A more detailed description of these new mutants and their transcriptional efficiencies *in vivo* and *in vitro* is in preparation (Lee, Concino and Weinmann, unpublished).

on the TATA box or the UPE. The conclusions from the experiments with DMS are similar to those with DNAse I. The -58 G is protected from DMS not only in wild-type Ad 2 MLP and in the point mutants, which do not reduce transcription efficiency, but also in the TATA box down mutants. However this site is not protected in the TA-56 down mutant. Together, the results indicate that binding to the TATA box is independent from binding to UPE of Ad 2 MLP and vice versa. This could imply either two different protein factors or two different domains of one very large protein are involved.

Our results together with results published by other authors, localize the region responsible for transcription efficiency in the Ad2 MLP *in vitro* to nucleotides -52 to -60, focussed at -56. The mutants from -41 to -51 and from -61 to -68 rarely affect transcription efficiency (maximal 10%). However the mutants within -52 to -60 reduce transcription efficiency significantly, for example, TA-56 by 50% (Fig. 8), GA-55, GA-57 by 60% (14). Hen *et al*. (15),

who analyzed the mRNA level transcribed from microinjected Ad 2 DNA deletion mutants, have suggested that the sequences around -62, which share homology with the rabbit β-globin gene from -60 to -69, might be important for transcription. Our results with point mutations, together with the results of Yu and Manley (14), show that none of the mutants tested within this region (CG-61, Fig. 8 and GA-66, GA-68, not shown, double mutant GA-62/GA-63), (15) affect in vitro transcription. This discrepancy could result from differences between in vivo and in vitro systems or between Ad 2 MLP and globin. Recent results of Lewis and Manley demonstrate that sequences between -66 and +7 are sufficient for efficient expression of the Ad 2 MLP in HeLa and 293 cells (23) in vivo.

While this manuscript was in preparation two reports presented data on the interaction between partially purified protein fractions required for transcription and the UPE and TATA box of the Ad2 MLP (24, 25). Sawadogo and Roeder have shown that the protein(s) binding to UPE and TATA box could be separated by heat stability and chromatography. We also observed that the TATA box binding protein is less stable than the UPE binding protein. Although both proteins bind independently, as shown here, a cooperative stabilization has been shown when both TATA box (TF II D) and UPE binding protein are bound simultaneously (24). This can be easily visualized with our footprinting results (see Figure 8) which suggest that an overlap between the two proteins occurs between nucleotides -50 to -45.

Comparison of the protected region on Ad 2 MLP with those observed in some other genes transcribed by polymerase II reveals many similarities (4, 7, 8). For example, in the SV40 early promoter the region between -40 and -100 from the start site is found protected by a cellular factor (4). In Drosophila, one of the transcription factor binds the sequences from -40 to +30 of histone 3 and actin gene promoter (7) and another factor recognizes the sequences from -40 to -100 in the heat-shock gene (8).

Our in vitro footprinting results show that protection in the TATA box region is distinct from that of the UPE region. The role of those regions is not completely clear. It is possible that the TATA box region is a binding site for a transcription factor common to most polymerase II genes, and the UPE region might be involved in transcriptional specificity of gene families controlled by common factors. Further analysis of these upstream regions in vivo as well as in vitro could shed more light on the mechanism underlying eukaryotic gene regulation at the transcriptional level.

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