Identification and Characterization of an Adenovirus 2 Major Late Promoter CAP Sequence DNA-binding Protein*

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DNase I footprint analysis of the core adenovirus 2 (Ad2) major late promoter (MLP) has revealed distinct patterns of protection corresponding to the assembly of transcription components during transcriptional initiation (VanDyke, M. W., Sawadogo, M., and Reeder, R. G. (1989) Mol. Cell Biol. 7, 3371–3379). By using partially purified transcription factors, DNase I protection over the TATA box element and the CAP sequence was attributed to the binding of a single factor, TFIIID. We have determined, however, that protection of the CAP region results from the binding of a novel factor, designated CAP-site binding factor (CBF), which is chromatographically and functionally distinct from TFIIID.

DNase I footprint analysis and gel electrophoresis mobility shift competition assays confirm that distinct polypeptides bind to the Ad2 MLP upstream promoter sequence, TATA box, and CAP sequences. When the CAP sequence is mutated, transcriptional activity of the Ad2 MLP is reduced both in vitro and in vivo. The decrease in transcriptional activity correlates with decreased CBF binding activity. Nuclear extracts depleted of CBF also exhibit reduced Ad2 MLP transcriptional activity. The addition of DNA affinity purified CBF, free of TFIIID or major late transcription factor, restores the activity to control levels.

An understanding of the molecular mechanisms underlying eukaryotic gene expression has in large part relied on the development and use of in vitro transcription systems using exogenous DNA templates (1–3). These systems have allowed the identification of several general transcription factors, in addition to RNA polymerase II, that associate at the core promoter to allow basal levels of transcription (4–8). Additional trans-acting factors bind to specific upstream cis-acting DNA elements to stimulate basal activity and/or achieve temporal and tissue specific activity. The characterization and purification of the various components involved in transcription initiation has been difficult, however, due to their relatively low abundance, instability, and the complexity of their interactions with the DNA template and/or other factors.

The adenovirus 2 (Ad2) major late promoter (MLP) is among the most active of known promoters to be accurately recognized and transcribed using extracts from uninfected cells (1–3, 9, 10). Initial experiments examining the effects of mutations on transcriptional activity of the Ad2 MLP determined that sequences in and around the TATA box, centered at −28, were essential for a basal level of transcription (9, 11–16). Maximal activity required the presence of an upstream promoter element, centered at position −55 (12, 14, 17–22). Distinct promoter elements binding to the TATA box, TFIIID (8, 22–24), and to the upstream promoter sequence, MLTF (25, 26), USF (14, 27), or UEF (28), have been identified and isolated. These two factors appear to bind cooperatively to the MLP (14).

A region of the Ad2 MLP that has not been as well characterized encompasses sequences surrounding the CAP site at +1, the point at which transcription begins. This region does not display extensive homology with most other promoters. Mutations to sequences at and just downstream of the MLP (14). Distinct protein factors binding to the TATA box, TFIIID (14, 22–24), and to the upstream promoter sequence, MLTF (25, 26), USF (14, 27), or UEF (28), have been identified and isolated. These two factors appear to bind cooperatively to the MLP (14).

MATERIALS AND METHODS

Plasmids and DNA Fragments—An AvuII-HindIII fragment of the Ad2 MLP (−138 to +193, Fig. 1A) was modified with Avul linkers and cloned into the pSAS vector as previously described (36). Following complete digestion with Avul, a 347-bp fragment was purified and cloned into the bacteriophage M13mp19 in order to generate mutant clones (see below). The purified fragment was also digested with different restriction enzymes (Fig. 1A) to generate Ad2 promoters.
Ad2 MLP CAP Sequence DNA-binding Protein

MLP fragments of various sizes were used in DNA-binding assays and to clone into plasmids for in vitro functional assays. Digestion of the Avai fragment with Bal31 exonuclease was performed to generate a fragment encompassing the TATA box and CAP site of the MLP (~38 to +28 for use in methylation interference assays.) The ~38 to +28 fragment was modified with BamHI linkers and recloned into the pUC19 plasmid. The sequence of the new plasmid, pUC8(CAP-TATA-CAP), was confirmed by double-stranded dideoxy sequencing (37). All plasmids were purified by phenol/chloroform precipitation (PICS) and identified by agarose gel electrophoresis (38).

Single-stranded oligonucleotides were synthesized by phosphoramidite chemistry (Vega, Coder 300 DNA Synthesizer) and purified by PICS (39). Coding and non-coding strands of Ad2 MLP sequences were purified by phenol/chloroform DNA affinity chromatography. Double-stranded sequences were annealed and double-stranded DNA fragments were purified from 8% non-denaturing polyacrylamide gels.

Construction of Mutant DNA Fragments—Mutations to the Ad2 MLP CAP sequence were produced in sequential steps. Oligonucleotide-directed mutagenesis (Amersham Corp.) was used to generate a MLP mutation (Δ10-12) with a 3'-bo end at position +10 to +12 (5'-CCC-3' to 5'-TAG-3'). This created a new XbaI restriction endonuclease site (Fig. 1C). The 347-bp Δ10-12 mutant fragment was inserted into wild-type MLP DNA affinity chromatography and DNA affinity chromatography. Digestion of PCR8(A10-12) with XbaI, followed by self-ligation with mum gene nucleas (BRL) or ligation, or modification by DNA polymerase I (Klenow fragment) and ligation, generated the pUC8-caps612 and pUC8-caps1316 mutant plasmids, respectively (Fig. 1C). The sequences of all the mutated promoters were confirmed by double-stranded dideoxy DNA sequencing (37).

Preparation and Fractionation of K562 Nuclear Extracts—K562 cells were grown in IMEM medium supplemented with 5% calf serum. Cells were maintained at a density of 1 × 10^5 to 1 × 10^6 cells/ml. Nuclear extract was prepared according to the method of Dignam et al. (3). All procedures were performed at 4°C. Extract initially digested against buffer D (20 mM Tris-HCl, pH 7.9, 0.2 mM EDTA, 10% glycerol, 0.1 M KCl) was adjusted to a final concentration of 0.5 M KCl and subjected to TSK-DEAE column chromatography (EM Science) to remove contaminating endogenous DNA. Protein that did not bind was dialyzed overnight against buffer D and centrifuged (25,000 x g) at 4°C. The supernatant was dialyzed against cacodylate, pH 8.0, and 1 mM EDTA (46). Following a 2 min incubation at room temperature, the reaction was stopped by the addition of 200 μl of 10 μM Tris at pH 8.0, 10 mM DTT, and 10% glycerol. Partially methylated DNA was purified and used in a standard mobility shift assay (17). The protein-DNA complex as well as the free DNA band were excised from the gel. DNA was eluted from the gel slices, purified by PICS (48), cleaved at modified guanine residues with 1.0 M piperidine at 90°C for 30 min, and analyzed on denaturing 6% polyacrylamide gels.

DNA Mobility Shift and DNase I Footprint Assays—The DNA mobility shift assays were performed according to the method of Sambrook and Russell (47). Binding reactions were incubated 20 min at room temperature, in the presence of unlabeled competitor fragments where indicated. Poly-[d(1-C)] or herring sperm DNA was used as non-specific competitor. Samples were electrophoresed at 4°C on non-denaturing 4% polyacrylamide gels.

DNase I footprinting reactions were performed according to the method of Galas and Schmitz (48). The reactions were incubated at 4°C for 20-40 min. Samples were electrophoresed on denaturing 6% polyacrylamide gels.

The renaturation of CBF-binding activity—the renaturation of CBF-binding activity was determined by SDS-PAGE using a modification of the procedure of Hager and Burgess (50). Gel slices (5 mm) containing proteins resolved by SDS PAGE were homogenized with a small Kontes pestle and eluted overnight into 1 ml of elution buffer (50 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.1% SDS, 5 mM DTT, 150 mM NaCl, and 10% glycerol) and incubated with 5 μg of pUC8 DNA to optimize in vitro transcriptional activity. Assays using K562 nuclear extract (85 μg) were identical except pUC8 DNA was not added. DNA template at the indicated concentration and nucleotides (100 μM ATP, CTP, UTP, 10 μM GTP, and 5 μCi of [α-32P]GTP, Amersham Corp.) were added. The reaction mixtures were incubated at 30°C for 30 min. RNA synthesis was terminated with the addition of buffer containing 50 mM Tris-HCl, 0.2% SDS, 0.5 mM EDTA and, where indicated, a 217-bp 32P-labeled DNA fragment to act as an internal control for recovery. Proteinase K and carrier tRNA were added to each reaction (20 and 10 μg, respectively) and incubated at 37°C for 15 min. RNA products were purified by organic extraction and ethanol precipitation prior to electrophoresis on 4% denaturing polyacrylamide gels.

The effects of factors on DNA affinity binding were measured in a DNA affinity assay using a DNA sequence unrelated to the Ad2 MLP. This sequence encompassed the 5' region of the 5'2-A gene, approximately 600 bp upstream of the transcriptional start site (43). The depleted extracts were stored in liquid nitrogen.

Heat-treated nuclear extract (HTNE) was prepared by incubating 200 μl of K562 nuclear extract at 47°C for 15 min (24). Transcription assays using HTNE were incubated in a total volume of 20 μl (40-70 μg) crude extract (40-70 μg) and the indicated amount of DNA affinity purified CBF were added. Volumes were adjusted to a total volume of 30 μl with buffer D.

Transfection and Chloramphenicol Acetyltransferase Assay—A monolayer of 293 cells were grown and maintained in IMEM supplemented with 5% calf serum (Bio-Fluids). A total of 10 μg of supercoiled DNA, consisting of 3 μg of the test plasmid and 7 μg of pUC8 carrier DNA, was transfected into subconfluent cells in a 100-mm plate using calcium phosphate precipitation (44).

Extracts were prepared from 293 cells at 48 h following transfection and assayed for chloramphenicol acetyltransferase activity according to the method of Gorman et al. (45). Chloramphenicol acetyltransferase was measured at total volumes of 6 μl using 32P-labeled ATP. Protein was normalized for the amount of extracted protein [7 μg (reaction)]. [3]Chloramphenicol was spotted onto silica gel thin layer chromatography plates (EM Science) and developed in chloroform/methanol (95:5). Following autoradiography of dried plates, the monocysteinated and non-acetylated chloramphenicol spots were cut out from the plate and counted by liquid scintillation to quantify enzymatic activity.

Heat-treatment incorporation of K562 transfection extract into subconfluent cells in a 100-mm plate using calcium phosphate precipitation (44).

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and 6 M guanidinium HCl (Pierce Chemical Co.), and incubated at room temperature for 30-60 min. The guanidinium was removed by applying the resuspended protein to a Sephadex G-25 Quick Spin Column (Boehringer Mannheim) previously equilibrated with denaturation buffer that lacked guanidinium HCl. Protein fractions (10 µl) were tested for DNA binding activity by mobility shift assays.

RESULTS

DNase I Footprint Competition Assays Identify Distinct Factors Binding to the Ad2 MLP—Binding of K562 nuclear proteins to the Ad2 MLP was investigated by DNase I footprinting (49). Protection over three distinct regions of the promoter was observed (Fig. 2, lanes 3 and 8). A DNase I footprint over the upstream promoter sequence (UPS), which binds the major late transcription factor (MLTF) (14), extended from -67 to -45 on the coding strand and from -66 to -48 on the non-coding strand. A DNase I footprint in the region of the TATA box extended from -31 to -8 on the coding strand and from -31 to -6 on the non-coding strand. An altered DNase I digestion pattern downstream of the

![Diagram](http://www.jbc.org/)

**Fig. 1.** The Ad2 MLP and DNA fragments used in protein binding assays. A, Cis-regulatory sequences of the Ad2 MLP. A map of an Ad2 MLP fragment (-138 to +193) between the AvaII-HindIII restriction endonuclease sites is shown. Outlined are the DNase I protected regions, the UPS (-67 to -45), the TATA element (-31 to -8), and the CAP sequence (+1 to +23). Each of these domains is numbered relative to the transcription initiation site at +1. Restriction endonuclease digestion of the AvaII/HindIII fragment with XbaI and DdeI generates a 92-bp CAP-specific fragment (-12 to +80) used in mobility shift assays. Digestion of the fragment with DdeI generates a 217-bp fragment (-138 to +80) used in DNase I footprinting assays. Digestion of the fragment with AluI generates a shorter promoter fragment (-138 to +33) that was cloned into plasmids upstream of the chloramphenicol acetyltransferase gene for in vivo assays of the Ad2 MLP. B, double-stranded synthetic DNA fragments used in protein binding assays. The CAP-specific fragment encompasses Ad2 MLP sequences between -12 to +23. The TATA fragment contains sequences -45 to -13 and the UPS-specific fragment encompasses sequences between -91 to -41. The METH fragment is a nonspecific DNA fragment that was used as a negative control and corresponds to a eukaryotic methylase-binding site (72). All fragments were synthesized with AvaI ends for cloning purposes.

C, Ad2 MLP sequences between +1 and +23 are shown for the wild-type and mutant CAP-binding domain. The Δ10-12 mutant has a 3 bp change (underlined bases) that generated a new XbaI restriction enzyme site. The Δ8-12 mutant has a 5 bp deletion (TCTAG) between positions +8 to +12, and the Δ13-16 mutant contains the 3-bp mutation at positions +10 to +12 (underlined) and an insertion of an additional 4 bp (CTAG) at positions +13 to +16.
Fig. 2. Competition analysis of a CAP-binding factor using DNase I footprint assays. A, 217-bp AvaI/Ddel Ad2 MLP DNA probe (−138 to +80) was 32P-labeled at the 5′ end of the coding strand or the 3′ end of the non-coding strand. DNase I footprint assays (49) were performed in a total of 25 μl, which included 0.5 ng of probe (approximately 20,000 cpm/reaction), 0.5 μg of poly(dI-C) and 85 μg of K562 nuclear extract. Where indicated, a 250-fold molar excess of competitor fragment (Fig. 1B) was simultaneously added with the probe. The products of G+A chemical sequencing reactions were used as size markers (lanes 1 and 6). DNA probes were partially digested with DNase I following incubation in the absence (lanes 2 and 7) or presence (lanes 3 and 8) of K562 nuclear extract. A 250-fold molar excess of the CAP-specific competitor fragment (lanes 4 and 9) or TATA-specific competitor fragment (lanes 5 and 10) was present in the binding reaction. Brackets outline the footprint over the CAP, TATA, and UPS on each strand, and (·) identifies the major DNase I hypersensitive site in the CAP-binding region.

transcription initiation site, over the CAP sequence, was also detected. This footprint on the coding strand was characterized by hypersensitive sites at the boundaries of the protected region, +1 and +23, and a strong hypersensitive site at position +12. On the non-coding strand, a weak footprint appeared between +2 and +20 and was marked by a weak hypersensitive site at +10.

To determine if distinct proteins were involved in the protection of these regions, DNA competitor fragments specific for the CAP region or the TATA box element (Fig. 1B) were added to the binding reaction. The TATA-specific DNA fragment abolished the footprint over the TATA box without disturbing the pattern over the CAP sequence (Fig. 2, lanes 5 and 10). Likewise, the CAP-specific competitor fragment competed for the footprint over the CAP sequence without affecting the protection over the TATA box sequence (lanes 4 and 9). Protein binding to the UPS on either the coding or non-coding strand was unaffected by the presence of either the TATA or CAP competitor fragments (lanes 3–5 and 8–10). These observations were confirmed in mobility shift assays with crude nuclear extract and an Ad2 MLP CAP sequence probe (−14 to +21) (data not shown). Protein-DNA complexes formed that were specifically competed only by the CAP sequence competitor fragment. These data identify a protein in crude nuclear extract that recognizes the CAP sequence of the Ad2 MLP. We designate this protein the CBF. Oligonucleotide competition analysis distinguishes CBF from TFIIID, which recognizes the TATA box element, and MLTF, which binds to the UPS.

The Chromatographic Behavior of CBF Is Distinct from TFIIID and MLTF—To compare further the properties of the Ad2 MLP DNA-binding proteins, CBF was partially purified from K562 nuclear extract. Sequential DEAE-anion exchange chromatography, phosphocellulose cation exchange chromatography, and DNA affinity chromatography were employed (Fig. 3A). CBF binding activity was detected by mobility shift assays in the PC 0.3 M KCl phosphocellulose (PC) fraction (Fig. 3B). Specific CBF-DNA complexes were competed by the unlabeled CAP competitor fragment (compare lane 1 with lanes 2–4), but not by the same concentration of the TATA fragment (lanes 5–7) or a nonspecific DNA fragment (METH) (lanes 8–10). The PC 0.3 M KCl fraction also generated a DNase I footprint over the Ad2 MLP CAP region that was identical to the footprint in crude nuclear extract (Fig. 3C, compare lane 3 with 4 and 8 with 9).

The PC 0.3 M KCl fraction was then subjected to DNA affinity chromatography (“Materials and Methods”). The 1.0 M KCl fraction from the DNA affinity column generated a DNase I footprint over the Ad2 MLP CAP sequence (Fig. 3C, lanes 5 and 10) as well as specific protein-DNA complexes in mobility shift assays (data not shown). Competition experiments revealed that protection by DNA affinity purified CBF over the CAP sequence was competed only by the CAP sequence-specific competitor fragment (Fig. 3D, lane 4). The same concentrations of competitor fragment specific for the TATA box element or the nonspecific METH sequence were without effect (lanes 5 and 6).

Since the DNA fragment used for the footprint assay encompassed the UPS and the TATA box element, it was possible to follow the independent segregation of MLTF and TFIIID from CBF. A footprint over the TATA box element was generated in crude nuclear extract, but not in either the PC 0.3 M KCl fraction or the 1.0 M DNA affinity fraction (Fig. 3C, lanes 3–5 and 8–10). Similarly, although MLTF purified in the PC 0.3 M fraction (Fig. 3C, lanes 4 and 9), the footprint was significantly reduced in the 1.0 M DNA affinity fraction (Fig. 3C, lanes 5 and 10). These data illustrate that the binding activity of DNA affinity purified CBF is chromatographically distinct from that of MLTF or TFIIID.

Identification of CBF as an 85–95-kDa Polypeptide—To determine the size of the polypeptide responsible for CBF binding activity, the protein renaturation assay of Hager and Burgess (50) was performed. DNA affinity purified CBF was electrophoresed by SDS-PAGE along with molecular weight standards. Proteins contained in gel slices were eluted, denatured, renatured, and analyzed for CBF binding by mobility shift assays (Fig. 4). A specific CBF-DNA complex was generated by proteins eluting from the gel slice corresponding to the molecular mass range of 85–95 kDa (Fig. 4, lane 4).

Mutations within the Ad2 MLP CAP Sequence Affect CBF Binding Activity—To identify close contact points between CBF and the Ad2 MLP CAP sequence, a methylation interference assay was performed (Fig. 5A). Partially methylated Ad2 MLP DNA fragments were incubated with the PC 0.3 M KCl CBF protein fraction under conditions for standard mobility shift assays. DNA molecules containing methylated
Fig. 3. DNA binding properties of purified CAP binding factor. A, Scheme for the partial purification of CBF as described under "Materials and Methods." B, a standard DNA mobility shift assay (47) was performed using a radiolabeled CAP-specific probe, -12 to +80, (Fig. 1A), as described under "Materials and Methods." Each reaction contained a total of 25 μl, including 0.5 ng of probe (20,000 cpm/reaction), 1 μg of poly[d(I-C)] and 4.5 μg of the PC 0.3 M KCl fraction. Bound identifies specific protein-DNA complexes (lane 1). Where indicated, a 10-, 50-, or 100-fold molar excess of the CAP, TATA, or METH competitor fragments (Fig. 1B) were added into the binding reaction. C, DNase I footprint assays were performed as in Fig. 2, with CAP protein at different stages of purification. Nonspecific competitor DNA (poly[d(I-C)]) was not present in reactions using DNA affinity purified CBF. The 217-bp 32P-labeled probe for either the coding or non-coding strand of the MLP was incubated in the absence of protein (lanes 2 and 7), or with 100 pg of crude K562 nuclear extract (lanes 3 and 8, 50 pg of the PC 0.3 M KCl fraction (lanes 4 and 9) or 2 μg of the 1.0 M DNA affinity fraction (lanes 5 and 10). Products of G+A chemical sequencing reactions were used as markers (lanes 2 and 7). Brackets outline the protected CAP, TATA, and UPS regions and (.) identifies DNase I hypersensitive sites at the CBF footprint. D, DNase I footprint assays were performed as in panel C, with the Ad2 MLP-coding strand. Probes were incubated without protein (lane 2), or with 2 μg of the 1.0 M KCl DNA affinity fraction (lane 3-6), containing 100-fold molar excess of the CAP, TATA, or METH competitor fragments where indicated.
**Fig. 4. Renaturation of gel-purified CBF.** DNA affinity purified CBF (4 μg) was electrophoresed by SDS-PAGE, and the proteins from individual gel slices were eluted, renatured, and assayed for DNA-binding activity as described under "Materials and Methods." Reactions contained the Ad2 MLP CAP sequence probe (−14 to +21). The load contained the 1.0 M KCl DNA affinity fraction prior to SDS-PAGE (lane 1). Molecular weights denoted above the gel refer to the location of the molecular weight protein standards relative to the gel slice.

Guanine residues that interfered with protein binding are excluded from forming complexes. Following cleavage of modified residues, those residues critical for binding are under-represented in the bound DNA as compared with the free DNA molecules. Methylation at the guanine residues at +11 on the coding strand (Fig. 5A, compare lanes 2 and 3) and at +10 and +12 on the non-coding strand (compare lanes 5 and 6) interfered with the binding of CBF to the Ad2 MLP.

Mutations within the CBF-binding domain were generated based on the above results (Fig. 1C and "Materials and Methods"). The binding of CBF to the wild-type and the Δ10-12 and i13-16 mutated MLP fragments was first analyzed using DNase I protection assays (Fig. 5B). Digestion patterns showed that the wild-type CAP fragment bound CBF in crude nuclear extract and in the PC 0.3 M KCl fraction (lanes 2 to 4). A footprint over the CAP sequence was not generated with these proteins on either the Δ10-12 or the i13-16 mutant fragments (Fig. 5B, lanes 5 to 10).

By using wild-type and mutated CBF recognition sequences in mobility shift assays, CBF-DNA complexes were identified only on the wild-type CAP sequence probe (Fig. 5C, lanes 1–3). Specific complexes were not formed on probes for the Δ10-12 mutant (lanes 4–6), the d8-12 mutant (lanes 7–9) or the i13-16 mutant (lanes 10 and 11). Similarly, competition experiments demonstrated that complexes formed by DNA affinity purified CBF on the wild-type CAP sequence probe, were efficiently competed by a fragment containing the wild-type CAP sequence, but not by as much as a 100-fold molar excess of fragments containing the Δ10-12, d8-12, or i13-16 mutations within the CAP sequence (data not shown). Non-specific protein-DNA complexes (NS) were formed on the wild-type and mutant DNA probes (Fig. 5C) and were competed by all the competitor fragments (data not shown).

**In Vitro and in Vivo Transcriptional Analysis of Ad2 MLP Templates Mutated within the CBF-binding Domain—To es...**

**Fig. 5. Binding characteristics of CBF to the wild-type Ad2 MLP fragment and fragments mutated within the CBF-binding domain.** A, A 5' 32P end-labeled probe was prepared for either the coding or non-coding strand of the Ad2 MLP from pUC66(TATA-CAP) (see "Materials and Methods"). Plasmid was first digested with...
The transcriptional activity from both the d8–12 mutant and the i13–16 mutant was 20–30% of wild-type levels. This reduction in activity correlated with the inability of CBF to bind to these mutant CAP sequences (Fig. 5). In contrast, the Δ10–12 mutant revealed only a minimal reduction in the transcriptional efficiency at any template concentration, with an overall activity of approximately 80% of wild-type levels. This result was inconsistent with the data from mobility shift and DNase I footprint assays, which demonstrated that CBF did not bind to the Δ10 12 mutation (Fig. 5). To resolve this contradiction, mobility shift assays were performed with wild-type and mutant CAP sequence probes under conditions in which the ionic strength was comparable to that in transcription reactions (Fig. 6C). At increasing KCl concentrations a specific complex with the Δ10–12 probe was observed (lanes 5–8), whereas specific binding was not observed with either the d8–12 or i13–16 mutant probes (lanes 9–16). The upper band (Bound) represented the specific CBF-DNA complex as determined by competition experiments (data not shown). Thus, the higher binding affinity of the Δ10–12 mutation for CBF in comparison to the d8–12 and i13–16 mutations was consistent with its relatively weak effect on in vitro transcription.

To assess the promoter strength in vivo, wild-type and mutated promoters were fused to the chloramphenicol acetyltransferase gene of E. coli and enzymatic activity determined (Fig. 7). The wild-type Ad2 MLP chloramphenicol acetyltransferase construct was transfected in parallel reactions and for each mutant the conversion of unacetylated [14C]chloramphenicol to the acetylated form was calculated relative to wild-type levels. A typical autoradiogram is shown in Fig. 7A, and the average of three independent experiments is presented in Fig. 7B. Chloramphenicol acetyltransferase expression was decreased to 52% of wild-type levels for the Δ10–12 mutant, 38% for d8–12, and to 69% for i13–16. These experiments demonstrate that the wild-type CBF-binding domain is required for optimal Ad2 MLP activity in vitro and in vivo.

DNA Affinity Purified CBF Rescues Transcriptional Activity to CBF-depleted Nuclear Extracts.—To correlate directly CBF binding and functional activity, DNA affinity purified CBF was assayed in nuclear extracts that were depleted of CBF by the CAP-specific DNA affinity resin (see “Materials and Methods”). Mobility shift assays were used to monitor depletion of CBF (Fig. 8A). CBF-DNA complexes were generated in control extract (Bound, lane 1), but not with the same probe in depleted extract (lane 2). Protein-DNA complexes were generated in both control and depleted extracts with a UPS-specific probe (Fig. 8A, lanes 3 and 4). These results demonstrate that CBF was depleted by the CAP sequence DNA affinity resin, but other regulatory proteins, for example MLTF, were not.

The transcriptional activity from the wild-type Ad2 MLP template in CBF-depleted extracts showed a 3-fold reduction compared with the levels in control extracts (Fig. 8B, compare lanes 1 and 2). The addition of DNA affinity purified CBF resulted in rescue of transcriptional activity to control levels (Fig. 8B, lanes 3–6). In contrast, transcriptional activity in an extract depleted by a DNA affinity resin containing nonspecific sequences unrelated to the Ad2 MLP was not rescued by the addition of CBF (Fig. 8B, lanes 7–10).

To eliminate the possibility that DNA affinity purified CBF used in the transcription rescue assay contained other stimulatory factors, such as MLTF, mobility shift assays were performed. CBF-DNA complexes formed only on a probe specific for the CAP sequence; whereas, complexes did not form on UPS-specific probes (Fig. 8C, lanes 1 and 2). Additionally, to test for the presence of TFIIID in the DNA affinity purified CBF, a H7NE was prepared in which TFIIID was inactivated (24). Specific transcription from the Ad2 MLP was abolished by this treatment (Fig. 8D, compare lanes 1 and 9). The activity was efficiently rescued, however, by the addition of the PC 1.0 M KCl fraction, containing TFIIID (24) (Fig. 8D, lanes 6–8). In contrast, DNA affinity purified CBF was not able to substitute for TFIIID to rescue transcriptional activity to the heated extract (Fig. 8D, lanes 3–5).

DISCUSSION

The transcriptional activity of the adenovirus 2 major late promoter has been shown to be regulated by trans-acting factors present in cellular extracts from uninfected cells. The core promoter of the Ad2 MLP has been described as consisting of the TATA box element and the associated CAP site sequences. These elements interact with components of the basic transcriptional machinery employed by most class II genes to determine the basal levels of promoter activity. A distinct protein, TFIIID, binds to the TATA box element (8, 22–24). Further upstream is the upstream promoter sequence that is required for optimal transcription from the Ad2 MLP. The UPS is bound by the major late transcription factor, also
known as USF or UEF (14, 25–28). These DNA sequences have been extensively characterized, and the factors that recognize them have been purified. Less information is available, however, regarding the role of the DNA at and just downstream of the transcription initiation site. In this report, a novel DNA element, the CAP sequence, has been identified downstream of the transcription initiation site. This element binds a previously unidentified protein, designated the CAP-site-binding factor, that is required for optimal transcriptional activity of the Ad2 MLP.

Mutations at and downstream of the CAP site show that these sequences are required for optimal activity (8, 16–20, 29, 51, 52). The adenovirus 5 E1a promoter required sequences downstream of the TATA box to +20 (53), whereas sequences to +53 were required for Ad2 early region EIII promoter activity (54). Mutations to DNA sequences between −10 to +7 of the mouse α-globin gene (55), +5 to +15 of the herpes simplex virus thymidine kinase gene (56, 57), and −5 to +5 of the adenovirus E1b promoter (58), markedly decreased the efficiency of in vitro transcription. Further, mutations to the silk fibroin gene promoter (59) and the long terminal repeat of the avian sarcoma virus (60), demonstrated that efficient transcription required sequences with the 3′ boundary at +6 for the silk fibroin gene and at +19 for the avian sarcoma virus long terminal repeat. Recently, novel DNA elements at the CAP site of the human gastrin gene (−17 to +57) and the terminal deoxynucleotidyl transferase gene (−6 to +11) were identified (61, 62). The element within the gastrin gene appears to be a cell-specific regulatory element that may function in conjunction with upstream elements to bring about efficient transcription. The terminal deoxynucleotidyl transferase element (termed the initiator) shows strong sequence similarity to the Ad2 MLP initiation site and directs accurate basal transcription from the terminal deoxynucleotidyl transferase gene.

Although transcriptional efficiency of these promoters appears to depend on the sequences surrounding the initiation site, factor binding to CAP site sequences has been identified for only a limited number of promoters (31–34). In this report, conventional DNase I footprinting and mobility shift assays have mapped the binding of CBF to DNA sequences immediately downstream of the Ad2 MLP initiation site. Compe-
tion experiments using crude and partially purified CBF confirmed that the protein was distinct from MLTF, binding to the Ad2 MLP UPS, and from TFII D, binding to the TATA box element. The DNA-binding properties of the partially purified protein showed that only the CAP sequence competed specifically and efficiently for complexes in mobility shift assays or the DNase I footprint over the CAP sequence (Fig. 3B and D). DNA-binding activities analyzed throughout fractionation demonstrated that MLTF copurified with CBF in the PC 0.3 M KCl fraction; however, the proteins were separated following DNA affinity chromatography (Fig. 8C). Similarly, the segregation of TFII D and CBF was consistent with a recent report in which TFII D was purified (24). TFII D eluted from phosphocellulose columns in buffers containing 0.85 M KCl, whereas in our chromatography scheme CBF eluted in buffers containing 0.3 M KCl. In addition, a denaturation/renaturation assay determined that polypeptides with a molecular mass of approximately 90 kDa contained specific CBF binding activity. Thus, CBF appeared to be distinct from TFII D and MLTF, which have Mr = 120,000 (8) and 43,000 (26, 63), respectively.

It has been suggested that a footprint spanning the TATA box and distal CAP region of the Ad2 MLP (−45 to +85) was the result of TFII D binding (24). This hypothesis is open to question, however, for several reasons. Methidiumpropyl-EDTA-Fe(II) footprinting revealed that specific TFII D interactions were limited to the TATA box element (−32 to −21) (14). Moreover, the downstream extension of the Ad2 MLP footprint (to +35) generated by a partially purified TFII D protein fraction was not detected by nonspecific interactions (24). Footprint analysis of this fraction showed only weak to nondetectable interactions downstream of the initiation site of several other promoters (24). In addition, yeast TFII D, which functionally substitutes for mammalian TFII D, generates a DNase I footprint that is limited to the TATA box consensus sequence (64, 65). Interestingly, when TFII D was present in conjunction with the other components of a functional preinitiation complex, an extended footprint was observed downstream of the initiation site of the MLP (23) and the adenovirus E4 promoter (66). A general model has been proposed, in which TFII D interacts with promoter specific upstream activator proteins, resulting in the sequence independent binding of TFII D to downstream sequences. Although it is likely that these protein-protein interactions occur to alter TFII D binding, it cannot be ruled out that an additional component of the transcription complex may directly interact with downstream promoter regions. In fact, the footprint on the E4 promoter generated by TFII D and the upstream activator protein, ATF, was altered when all the basic transcription components were present. Further, the
nonspecific interaction of TFIID to downstream Ad2 MLP sequence may have masked the binding of a specific factor since adenoviral sequences extended only to +10.

A functional role for CBF was demonstrated by analysis of templates mutated within the CBF-binding domain based on methylation interference assays. In vitro chloramphenicol acetyltransferase assays showed a decrease in the transcriptional activity of three different mutant promoters. In vitro transcription assays showed that activity was significantly reduced from the d8–12 and i13–16 mutations, and only slightly reduced from the a10–12 mutation. Differences in the transcriptional efficiencies of each of the mutated promoters may be due to the inherent differences in each system. The in vitro data reflect the actual levels of RNA synthesis from isolated DNA fragments, whereas chloramphenicol acetyltransferase assays are a direct measure of stable enzyme activity after the mRNA has been transcribed, processed, transported, and translated. Alternatively, the relative levels of transcription may reflect different concentrations of specific transcription factors present in the cell types used for each assay. The decreased transcriptional activity of the d8–12 and i13–16 mutations was consistent with the inability of these mutants to bind purified CBF. This establishes the sequence specificity of CBF binding and implies that CBF acts as a positive regulatory factor through its interaction with the CAP sequence element of the Ad2 MLP. Mobility shift assays performed under conditions that closely parallel those of the in vitro transcription system demonstrated a low affinity of CBF for the A10–12 mutant. This may explain the higher levels of activity from this mutant in the in vitro system.

A more direct evaluation of CBF activity used extracts depleted of CBF by the CAP sequence DNA affinity resin. Following depletion, transcriptional activity of the Ad2 MLP was reduced 3-fold. Addition of DNA affinity purified CBF rescued activity of CBF-depleted extracts to control levels. Extracts depleted with a nonspecific DNA affinity resin or inactivated of TFIID activity showed a decrease in Ad2 MLP transcriptional activity. These results appear to be due to the depletion of rate-limiting factors other than CBF. Hence, the transcriptional activity in these extracts was not rescued by the addition of CBF (Fig. 8, B and D). Therefore, we conclude that DNA affinity purified CBF contains a novel protein with specific binding activity for the Ad2 MLP CAP sequence. This factor is required for optimal Ad2 MLP transcription. In addition, it is chromatographically and functionally distinct from TFIID and ML1/F.

Although CBF is a cellular factor present in uninfected cells, whether other promoters utilize CBF is unknown. Most of the promoters that require wild-type sequences in the CAP region do not display any striking sequence similarity with either the Ad2 MLP CAP domain or with each other. This may suggest that a family of functionally related factors exists, with different sequence specificities for DNA binding. Alternatively, CAP-binding proteins may recognize a different regulatory factor through its interaction with the CAP sequence. Other transcriptional factors present in the cell types used for each assay. The relative levels of transcription may reflect different concentrations of specific transcription factors present in the cell types used for each assay. The decreased transcriptional activity of the d8–12 and i13–16 mutations was consistent with the inability of these mutants to bind purified CBF. This establishes the sequence specificity of CBF binding and implies that CBF acts as a positive regulatory factor through its interaction with the CAP sequence element of the Ad2 MLP. Mobility shift assays performed under conditions that closely parallel those of the in vitro transcription system demonstrated a low affinity of CBF for the A10–12 mutant. This may explain the higher levels of activity from this mutant in the in vitro system.

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Sawadogo, M., and Roeder, R. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4826-4830.
28. Moncollin, V., Miyamoto, N. G., Zheng, X. M., and Egly, J. M. (1986) EMBO J. 5, 2577-2584.
29. Concino, M. F., Lee, R. F., Merryweather, J. P., and Weinmann, R. (1984) Nucl. Acids Res. 12, 7423-7433.
30. Albrecht, G., Devaux, B., and Kedinge, C. (1988) Mol. Cell Biol. 8, 1534-1539.
31. Cai, H., and Luse, D. S. (1987) Mol. Cell Biol. 7, 3371-3379.
32. Parks, C. L., Banerjee, S., and Spector, D. J. (1988) J. Virol. 62, 54-67.
33. Cordingly, M. G., Riegel, A. T., and Hager, G. L. (1987) Cell 48, 261-270.
34. Stenlund, A., Bream, G. L., and Botchan, M. R. (1987) Science 236, 1666-1671.
35. Wu, F. K., Garcia, J. A., Harrich, D., and Gaynor, R. B. (1988) EMBO J. 7, 2117-2129.
36. Thompson, J. A., Garfinkel, S., Cohen, R. B., and Safer, B. (1987) Biochromatography 2, 190-176.
37. Chen, E. Y., and Seeberg, P. H. (1985) DNA 4, 165-170.
38. Thompson, J. A. (1986) Biochromatography 1, 68-80.
39. Thompson, J. A. (1986) Biochromatography 1, 22-32.
40. Marius, M. G., and Morris, N. R. (1973) J. Bacteriol. 114, 1143-1150.
41. Geier, G. E., and Modrich, P. (1979) J. Biol. Chem. 254, 1408-1413.
42. Kadonaga, J. T., and Tjian, R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5889-5893.
43. Jacob, W. F., Silverman, T. A., Cohen, R. B., and Safer, B. (1989) J. Biol. Chem. 264, 20372-20384.
44. Graham, F. L., and van der Eb, A. J. (1973) Virology 52, 456-467.
45. Gorman, C. M., Moffat, L., and Howard, B. H. (1982) Mol. Cell Biol. 2, 1044-1051.
46. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499-560.
47. Strauss, F., and Varshavsky, A. (1984) Cell 37, 889-901.
48. Thompson, J. A. (1987) Biochromatography 2, 1-18.
49. Galas, D. J., and Schmitz, A. (1978) Nucl. Acids Res. 5, 3157-3170.
50. Hager, D. A., and Burgess, R. R. (1980) Anal. Biochem. 109, 76-86.
51. Mansour, S. L., Grodzicker, T., and Tjian, R. (1986) Mol. Cell Biol. 6, 2684-2694.
52. Cohen, R. B., Yaman, L., Thompson, J. A., and Safer, B. (1988) J. Biol. Chem. 263, 10377-10385.
53. Hearing, P., and Shenk, T. (1983) J. Mol. Biol. 167, 809-822.
54. Lee, D. C., Roeder, R. G., and Wold, W. S. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 41-45.
55. Tallington, C. A., and Leder, P. (1982) Nature 298, 192-195.
56. McKnight, S. L., and Kingsbury, R. (1982) Science 217, 316-324.
57. Coen, D. M., Weinheimer, S. P., and McKnight, S. L. (1986) Science 234, 53-59.
58. Wu, L., Rosser, D., Schmidt, M. C., and Berk, A. (1987) Nature 326, 512-515.
59. Tsuchimoto, Y., Hirose, S., Toda, M., and Suzuki, Y. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4838-4842.
60. Mitsialis, S. A., Manley, J. L., and Guntaka, R. V. (1983) Mol. Cell Biol. 3, 311-318.
61. Theill, E., Wiborg, O., and Vuust, J. (1987) Mol. Cell Biol. 7, 4329-4336.
62. Smale, S. T., and Baltimore, D. (1989) Cell 57, 103-113.
63. Sawadogo, M., Van Dyke, M. W., Gregor, P. D., and Roeder, R. G. (1988) J. Biol. Chem. 263, 11985-11993.
64. Buratowski, S., Hahn, S., Sharp, P. A., and Gursene, L. (1988) Nature 334, 57-42.
65. Cavallini, B., Huet, J., Plassat, J. L., Sentenac, A., Egly, J. M., and Chambon, P. (1988) Nature 334, 77-80.
66. Horikoshi, M., Hie, T., Lin, Y. S., Green, M. R., and Roeder, R. G. (1988) Cell 54, 1033-1042.
67. Hauber, J., and Culley, B. R. (1988) J. Biol. Chem. 69, 679-679.
68. Garcia, J. A., Wu, F. K., Mitsuyasu, R., and Gaynor, R. B. (1987) EMBO J. 6, 3761-3770.
69. Garcia, J. A., Hartir, D., Soultanakis, E., Wu, F., Mitsuyasu, R., and Gaynor, R. B. (1988) EMBO J. 8, 765-778.
70. Ziff, E. B., and Evans, R. M. (1978) Cell 15, 1463-1475.
71. Godon, C. R., and Rossel, W. C. (1973) Nucl. Acids Res. 11, 21-26.
72. Wang, R., Zhang, X., and Ehrlich, M. (1986) Nucl. Acids Res. 14, 1599-1614.
73. Morgan, R. A., Christy, R. J., and Huang, R. C. (1988) Develop-ment 102, 23-30.
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S Garfinkel, J A Thompson, W F Jacob, R Cohen and B Safer

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