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A Cellular DNA-Binding Protein That Activates Eukaryotic Transcription and DNA Replication

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Summary

Transcription factor CTF, which is responsible for selective recognition of eukaryotic promoters that contain the sequence CCAAT, was purified to apparent homogeneity by sequence-specific DNA affinity chromatography. Binding sites for CTF in the human Ha-ras and α-globin promoters were highly homologous to sequences recognized by nuclear factor I (NF-I), a cellular DNA-binding protein that is required for the initiation of adenovirus DNA replication in vitro. To determine the relationship between CTF and NF-I, we compared the biochemical properties of these two proteins. CTF and NF-I were found to be indistinguishable in polypeptide composition, DNA-binding properties, immunological cross-reactivity, and in vitro stimulation of DNA replication and transcription initiation. We conclude that CTF/NF-I can serve both as a transcription selectivity factor for RNA polymerase II and as an initiation factor for adenovirus DNA replication.

Introduction

Proteins that interact with specific DNA recognition elements are thought to contribute to the temporal and spatial restriction of important cellular processes such as replication and transcription. Studies of transcription have clearly established that eukaryotic protein-coding genes contain a complex array of *cis*-regulatory elements that mediate induced, repressed, or basal transcription rates. Although it is generally accepted that multiple classes of interactive DNA-binding proteins regulate gene expression in higher organisms, relatively few of the protein species that recognize RNA polymerase II promoter elements have been purified to homogeneity and analyzed biochemically. Similarly, few DNA-binding proteins involved in replication have been identified and fully characterized, in part because accurate replication from eukaryotic origins requires a complex assortment of protein factors. Nevertheless, the characterization of protein–DNA interactions at eukaryotic promoters and replication origins provides one of the few means by which the mechanisms of transcription and replication initiation can be studied.

Mutational analyses of eukaryotic promoters revealed the existence of a promoter element containing the sequence CCAAT, which is required for transcription of a class of cellular genes that includes many vertebrate globin gene families. The CCAAT element of the mouse β-globin promoter interacts with yet another promoter element that contains the sequence CCACACCCG (Dierks et al., 1983; Charnay et al., 1985; Myers et al., 1986), whereas the CCAAT element of the human α-globin promoter appears to function independently of other types of promoter elements (Mellon et al., 1981). The CCAAT promoter element is also interspersed with binding sites for the Sp1 transcription factor in the herpesvirus thymidine kinase (*tk*) gene (McKnight et al., 1984; Jones et al., 1985), and with heat-shock elements in the Xenopus and human *hsp70* genes (Bienz and Pelham, 1986; W. Morgan and R. Tjian, unpublished data). In vitro transcription studies revealed the existence in HeLa extracts of a protein, CTF (CCAAT-binding transcription factor), that recognizes the CCAAT domain of the *tk* promoter (Jones et al., 1985), and a factor has also been reported to bind to the mouse α- and β-globin promoter CCAAT sequences (Cohen et al., 1986).

Analysis of the binding of CTF to a variety of promoters revealed that the nucleotide sequence recognized by the protein matches a portion of the consensus recognition sequence previously established for the adenovirus replication protein, nuclear factor I (NF-I) (Nagata et al., 1983; Rawlins et al., 1984; Siebenlist et al., 1984; Gronostajski et al., 1985; Hennighausen et al., 1985; Nowock et al., 1985; deVries et al., 1985; Rosenfeld and Kelly, 1986; Schneider et al., 1986). NF-I is a cellular protein that is required for the initiation of adenovirus DNA replication in vitro and in vivo (Nagata et al., 1982; Rawlins et al., 1984; Hay, 1985; Pearson and Wang, 1985; Bernstein et al., 1986). Genetic and biochemical experiments have demonstrated that NF-I binds with high affinity to a specific sequence element within the adenovirus origin of replication and that binding is absolutely required for efficient initiation of replication (Guggenheimer et al., 1984; Rawlins et al., 1984; deVries et al., 1985; Leegwater et al., 1985). The essential components of the NF-I recognition sequence have been identified by comparison of the sequences of high-affinity binding sites from a variety of viral and cellular genomes (Rawlins et al., 1984; Siebenlist et al., 1984; Gronostajski et al., 1985; Hennighausen et al., 1985; Nowock et al., 1985; Rosenfeld and Kelly, 1986; Schneider et al., 1986), and by measurement of the affinity of NF-I sites containing single-base substitution mutations (deVries et al., 1985; Schneider et al., 1986; Rosenfeld et al., 1987). The consensus recognition sequence derived from these studies is TGG(A/C)N_5GCCAA. NF-I has been purified from crude HeLa cell fractions to near homogeneity by a two-step affinity procedure that involves chromatography on a matrix containing a high density of specific recognition sites (Rosenfeld and Kelly, 1986), and

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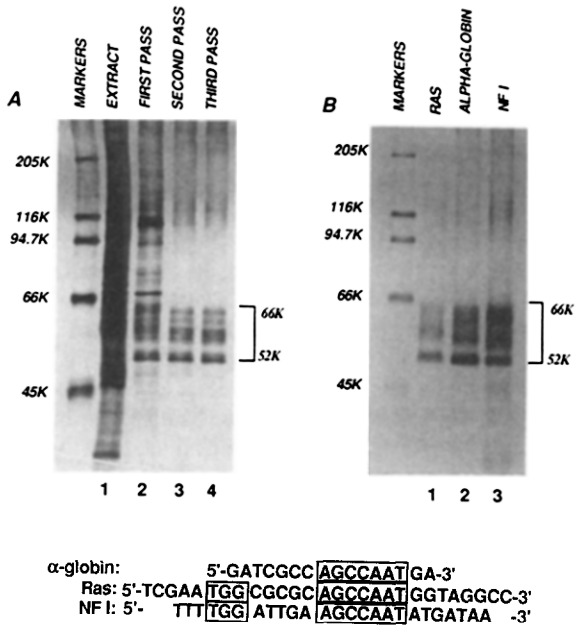

Figure 1. Purification of CTF by Sequence-Specific DNA-Affinity Chromatography

(A) Analysis of affinity-purified CTF fractions by 8% SDS–polyacrylamide gel electrophoresis and silver staining. Lane 1, protein fraction before purification with the Ha-ras affinity column, approximately 5 U (100 μg); lane 2, first-pass eluate, 100 U; lane 3, second-pass eluate, 100 U; lane 4, third-pass eluate, 100 U. One unit is defined as the amount of protein required to bind 10 fmol of the human α-1 globin promoter CTF-binding site with the DNAase I footprint technique. Molecular size markers were 167 ng each of myosin (rabbit muscle), 205K; β-galactosidase (E. coli), 116K; Phosphorylase b (rabbit muscle), 97.4K; bovine serum albumin, 66K; egg albumin, 45K; and bovine erythrocyte carbonic anhydrase, 29K. The migration of the BSA protein marker was anomalous with respect to size under these conditions (see Experimental Procedures).

(B) Comparison of CTF derived from the Ha-ras and α-globin affinity resins with NF-I derived from the adenovirus origin affinity resin. Lane 1, CTF purified with the Ha-ras column; lane 2, CTF purified with the human α-globin column; lane 3, NF-I purified with the adenovirus origin column. The molecular weight marker lane contained 50 ng each of the six reference proteins listed above.

The bottom diagram lists the sequences used in the construction of the different affinity columns; only the relevant portion of the 67 bp DNA fragment used to construct the NF-I column is shown (Rosenfeld and Kelly, 1986).

the purified protein was shown to consist of a family of polypeptides with molecular weights between 52 and 66 kd.

We report here the purification of CTF to apparent homogeneity by sequence-specific DNA affinity chromatography with CAAT-box elements from the human α-globin and Ha-ras genes. To test whether the different biochemical activities of CTF and NF-I are a function of the same protein species, we have carried out a direct comparison of the physical and biological properties of highly purified preparations of the two factors. We could detect no difference between CTF and NF-I by SDS–polyacrylamide gel electrophoresis, by several different assays of specific binding to promoters carrying the CCAAT element or to the adenovirus origin of DNA replication, or by assays that measure the ability of the two factors to stimulate adenovirus DNA replication in vitro or to activate the human α-globin promoter in a reconstituted transcription system. We conclude that CTF/NF-I consists of identical proteins that can function both as a transcription selectivity factor and as an initiation factor for DNA replication.

Results

Purification of CTF by Sequence-Specific DNA Affinity Chromatography

We previously reported the identification and partial purification of a transcription factor, CTF, from HeLa nuclear extracts (Jones et al., 1985). To purify CTF further, we applied the affinity chromatography technique of Kadonaga and Tjian (1986), in which relatively crude protein fractions are incubated with competitor DNA and passed over an agarose matrix containing synthetic oligodeoxynucleotides derived from high-affinity DNA-binding sites. Binding studies with various nuclear fractions enriched for CTF activity (estimated purity, 1%–2%; see Experimental Procedures) suggested that the distal upstream region (−300) of the human Harvey ras-1 (Ha-ras) gene and the promoter region (−70) of the human α-1 globin gene contain relatively high-affinity binding sites for CTF that would be suitable for affinity chromatography.

Short double-stranded oligodeoxynucleotides consisting of a 28 bp segment of the Ha-ras site and a sixteen bp subset of the α-globin CTF-binding site (Figure 1) were ligated and used to generate two distinct affinity resins. Both of these resins were found to retain CTF specifically from relatively crude protein fractions that were derived from HeLa nuclear extracts (see Experimental Procedures). Analysis of the affinity-purified CTF fractions by SDS–polyacrylamide gel electrophoresis and silver staining is shown in Figure 1A. A heterogeneous group of polypeptides was eluted after one pass of an S-300 gel-filtration fraction over the Ha-ras affinity resin. The first-pass eluate was, however, highly enriched for a set of proteins (52 to 66 kd) that was not prominent in the input fraction (Figure 1A, compare lanes 1 and 2). The second-pass eluate contained the 52–66 kd group of polypeptides, but none of the higher molecular weight contaminants that were present in the first-pass eluate (Figure 1A, compare lanes 2 and 3). Subsequent repeated chromatography over the Ha-ras resin yielded the same 52–66 kd group of proteins without detectable change in the relative polypeptide ratios (Figure 1A, compare lanes 3 and 4). An estimated 500-fold purification from the input S-300 gel-filtration fraction and a 30%–50% recovery of DNA-binding activity was obtained after two passes on the Ha-ras column.

A similar group of polypeptides was purified when the S-300 gel-filtration fraction was chromatographed on the α-globin affinity resin (Figure 1B, compare lanes 1 and 2). Moreover, the proteins eluted from the Ha-ras column were found to adhere specifically and quantitatively to the α-globin affinity column, and the entire group of polypeptides (52–66 kd), with the same relative stoichiometries, was eluted from the resin with a single high-salt step (data not shown). The α-globin resin was less efficient than the

resin prepared with the Ha-ras CTF-binding site because CTF fractions had to be passed four to five times over the α-globin column to obtain a purity comparable to that observed after only two passes on the Ha-ras column. As described below, this is most likely due to differences in the affinity of the proteins for the Ha-ras and α-globin sequences. The overall recovery of CTF-binding activity from a nuclear extract of HeLa cells was 25%–40%, with a yield of approximately 100 μg of protein from a 32 l prep ($1–2 \times 10^{10}$ cells).

Comparison of Biochemical Activities of CTF and Nuclear Factor I

We noticed that a potential NF-I recognition element, 5′-TGG(A/C)N_5GCCAA-3′ (Borgmeyer et al., 1984; Nowock et al., 1985; Hennighausen et al., 1985; Gronostajski et al., 1985; Rawlins et al., 1986; Rosenfeld et al., 1987), was in the Ha-ras CTF-binding site (outlined in boxes, Figure 1). Although only a portion (5′-GCCAA-3′) of this consensus was in the α-globin binding site, or in ligated multimers of the complementary oligodeoxynucleotides used in the α-globin column, the same set of DNA-binding proteins was specifically retained on the Ha-ras and α-globin columns. Furthermore, the set of DNA-binding proteins corresponding to CTF was similar in molecular weight to the family of related proteins previously purified as NF-I by Rosenfeld and Kelly (1986). Because these similarities suggested that CTF and NF-I might be structurally and functionally related, we carried out direct comparisons of the properties of independently purified CTF and NF-I protein fractions. The CTF fractions used in this report were purified with the Ha-ras and α-globin affinity columns following S-300 gel-filtration chromatography of the HeLa nuclear extract. The NF-I fractions were purified from a Bio-Rex 70 eluate of HeLa cell nuclear extracts by DNA recognition-site affinity chromatography (Rosenfeld and Kelly, 1986). This two-step affinity procedure includes chromatography on a matrix prepared from a plasmid that contains 88 copies of the adenovirus origin of DNA replication. CTF purification was monitored by binding to promoters that carry the CCAAT motif and stimulation of α-globin transcription in vitro, whereas NF-I was purified by binding to the adenovirus origin and stimulation of adenovirus DNA replication.

To compare the polypeptide composition of purified CTF and NF-I directly, approximately 500 ng of each protein was analyzed by electrophoresis on an 8% SDS–polyacrylamide gel followed by silver staining. The polypeptides purified by resins containing either the α-globin, Ha-ras, or adenovirus origin sequences were similar (Figure 1B, lanes 1–3), and consisted of a major group between 55 and 66 kd molecular weight, plus a doublet at approximately 52 kd molecular weight. The only other species reproducibly detected in fractions from all three columns was a broad band at approximately 120 kd, which was present in substantially lower molar amounts than any of the polypeptides in the 52–66 kd group. Although slight variations in the relative abundance of the 52 kd doublet were observed from different extracts, these differences were independent of the column resin used to isolate the protein. The yield of CTF with the Ha-ras and α-globin CCAAT resins was approximately one-third of that reported for NF-I (Rosenfeld and Kelly, 1986), which may be due to either the lower affinity of the CTF sites used in the affinity resins or to differences in the initial steps of the preparation and fractionation of the nuclear extract (see Experimental Procedures). Thus the group of cellular proteins that interacts specifically with a 16 bp subset of the α-globin CCAAT box and a 28 bp fragment containing the Ha-ras site strongly resembles the proteins that bind to the adenovirus NF-I site.

To determine whether CTF and NF-I recognize DNA in a similar manner, the DNAase I footprint patterns and relative affinities of each protein for several different sites were compared. The interaction of CTF (derived from either the Ha-ras or α-globin columns) and NF-I with the human α- and β-globin promoters was evaluated with the DNAase I footprint technique (Galas and Schmitz, 1978). Both CTF and NF-I generated identical 18 bp footprints on the β-globin promoter and 23 bp footprints on the α-globin promoter (Figure 2A), and protected a region that is asymmetrically positioned around the CCAAT sequence. Identical recognition sites for CTF and NF-I were also observed on the HSV1 *tk* promoter (Figure 2A) and the adenovirus origin of DNA replication (Figure 2B). CTF displayed at least a 20-fold higher affinity for the α-globin promoter than the β-globin CCAAT element, and the origin sequences were bound with at least a 5-fold higher affinity than the α-globin promoter sequences. The CTF-binding site on the HSV-*tk* promoter is located next to binding sites for the Sp1 transcription factor, and the binding site on the β-globin promoter lies adjacent to a site for a different transcription factor that recognizes the CCACACCCG promoter element (Figure 2A; P. Mitchell, W. Lee, and R. Tjian, unpublished data). Additional CTF-binding sites were observed on both the β-globin and HSV-*tk* promoters, including high-affinity distal binding sites in both promoters and a lower affinity binding site that maps at or near the β-globin TATA domain (data not shown). Moreover, the pUC9 vector of the adenovirus origin plasmid harbors a fortuitous CCAAT sequence that is recognized equally by the CTF and NF-I fractions. Thus both fractions contain binding activities that similarly discriminate between various high- and low-affinity CTF-binding sites, which strongly suggests that CTF and NF-I binding activities derive from the same protein species.

Protein–DNA interactions may also be evaluated with a variety of other techniques, some of which provide information that is not accessible by DNAase footprint analysis. For example, the gel mobility shift technique (Fried and Crothers, 1981; Garner and Revzin, 1981) can provide information on the apparent binding-site stoichiometry. Aliquots of CTF or NF-I were incubated with end-labeled DNA fragments containing the adenovirus origin, and the protein–DNA complexes were analyzed by electrophoresis on a 6% nondenaturing polyacrylamide gel followed by autoradiography. The resulting mobility shift patterns were identical for NF-I and CTF (Figure 2C). In contrast, the mobility of the control DNA fragment was not altered by either protein. It is also interesting to note that the pat-

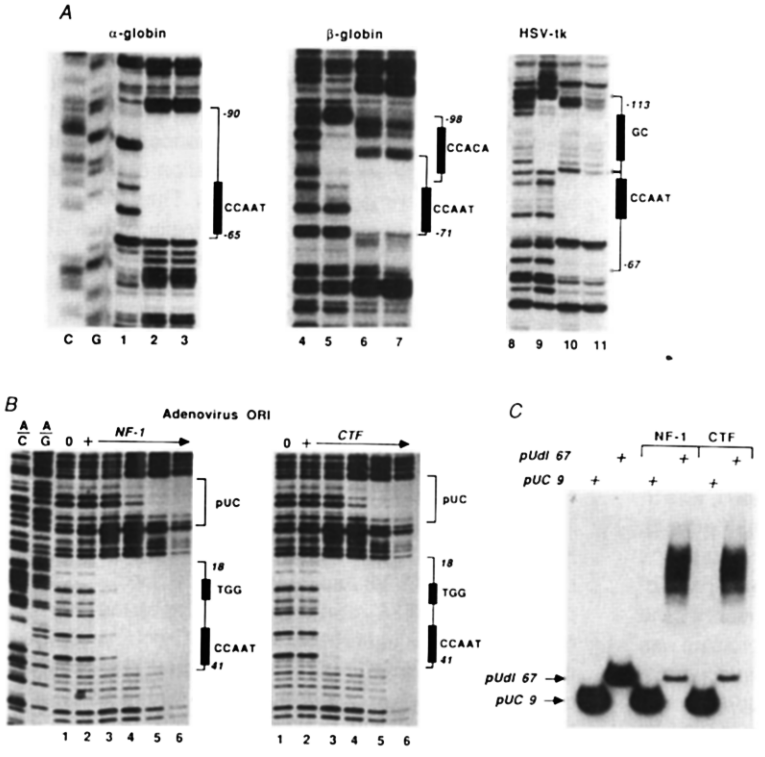

Figure 2. Comparison of the DNA-Binding Properties of CTF and NF-I

(A) DNAase I footprint analysis of CTF and NF-I interactions with CCAAT-box elements present in RNA polymerase II promoters. Binding to the α-globin promoter was carried out with a 610 bp NcoI–PstI promoter fragment that was 5′ end labeled at the NcoI site (+100). Other footprint probes include the NcoI–HinfI β-globin promoter fragment, 5′ end labeled at the NcoI site (+100); and the BglII–HindIII fragment of the pseudo-wild-type HSV-*tk* promoter, 5′ end labeled at the BglII site (+35). Lanes C and G, α-globin sequence markers; lanes 1, 4, and 8 show the control digestion pattern observed in the absence of added DNA-binding protein; lanes 2, 6, and 10 show the footprint pattern in the presence of 10 ng of NF-I; lanes 3, 7, and 11 show the footprint pattern observed with 10 ng of CTF (α-globin CTF, lane 3; Ha-Ras CTF, lanes 7 and 11). Lane 5 shows the footprint pattern on the human β-globin promoter with 20 ng of a distinct affinity-purified factor (P. Mitchell, W. Lee, and R. Tjian, unpublished data), and lane 9 shows the second distal GC-box footprint on the HSV-*tk* promoter observed with 40 ng of the affinity-purified Sp1 transcription factor (Kadonaga and Tjian, 1986; Briggs et al., 1986).

(B) DNAase I footprint analysis of CTF or NF-I interaction at the adenovirus origin of DNA replication. Footprint probe was the 157 bp BamHI–PvuII fragment of plasmid pUd 67, 5′ end labeled at the BamHI site. Footprint reactions were carried out with the following amounts of CTF (α-globin) or NF-I, as indicated in the figure: 0 ng, lane 1; 0.1 ng, lane 2; 0.4 ng, lane 3; 1.6 ng, lane 4; 6.4 ng, lane 5; 25 ng, lane 6. Lanes A/C and A/G are sequence markers. Sequence markers were prepared according to a modification of the Maxam and Gilbert protocol (Bencini et al., 1984). The binding site labeled pUC maps to a region between −14 and 2 with respect to the EcoRI site, 5′-TCGACCTGCAGCCAAG-3′, which is present in the pUC9 vector polylinker.

(C) Comparison of CTF and NF-I by electrophoretic mobility shift analysis. The radioactive DNA fragments used in the assay were a 157 bp BamHI–PvuII fragment from pUdI 67, which contains the adenovirus origin of DNA replication, and a 101 bp BamHI–PvuII control fragment from pUC9, which lacks the NF-I binding site. DNA fragments were incubated with or without NF-I (10 ng) or CTF (α-globin; 10 ng) and electrophoresed through a native polyacrylamide gel as described in Experimental Procedures.

tern of bands representing different protein–DNA complexes in the nondenaturing gel strongly resembles the polypeptide pattern observed by SDS–polyacrylamide gel electrophoresis of purified NF-I and CTF. Because the CTF and NF-I fractions contain a group of polypeptides with different molecular weights, the multiple bands observed in the presence of CTF or NF-I (Figure 2C) could be due to the binding of different numbers of promoters to DNA, to the binding of single polypeptides of distinct molecular weights, or to some combination of both the number and size of the polypeptides bound to DNA. It is therefore striking that in spite of the complexities involved in determining the gel mobility shift pattern, the final pattern observed is identical for CTF and NF-I.

Competition DNA-Binding Experiments

Because the affinity-purified fractions contain a group of proteins and not a single polypeptide species, we decided to test whether oligodeoxynucleotides containing the binding sequences of the α-globin and adenovirus NF-I sites would compete equally for the binding of CTF to each site. DNAase I footprint reactions were carried out with either α-globin or adenovirus origin DNA fragments in the presence of a wide range of competitor oligodeoxynucleotides. Both sequences competed efficiently for the binding of CTF to either the α-globin site or the adenovirus origin at competitor binding-site concentrations of 0.16 nM (origin binding sites) and 0.8 nM (α-globin binding sites; Figure 3). In contrast, control oligodeoxynucleotides that

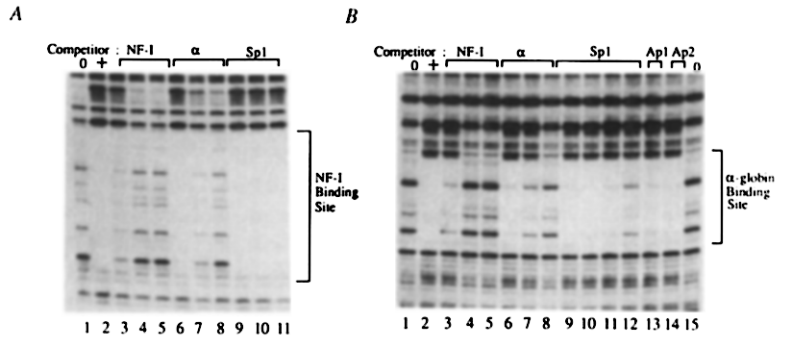

Figure 3. Competition Footprint Experiments

(A) Binding of affinity-purified CTF (Ha-ras) to the adenovirus origin NF-I binding site. Binding was carried out as described in Experimental Procedures; competitor DNA concentrations were 0 nm (lane 2), 0.16 nm (lanes 3, 6, and 9), 0.8 nm (lanes 4, 7, and 10), and 80 nm (lanes 5, 8, and 11) of the ligated oligodeoxynucleotides (as indicated above each lane).

(B) Binding of affinity-purified CTF (Ha-ras) to the α-globin CCAAT box. Competitor DNA concentrations in moles of specific transcription factor binding sites were 0 nm (lane 2), 0.16 nm (lanes 3, 6, and 9), 0.8 nm (lanes 4, 7, and 10), 80 nm (lanes 5, 8, 11, 13, and 14), and 400 nm (lane 12).

contain binding sites for unrelated DNA-binding proteins such as Sp1, AP-1, or AP-2 did not compete at binding-site concentrations as high as 80 nM. Thus we conclude that CTF and NF-I possess indistinguishable DNA-binding properties.

Immunological Properties of CTF and NF-I

To examine further the relationship between CTF and NF-I, we used a DNA fragment immunoprecipitation technique that is based on the recognition of protein–DNA complexes by antibodies directed against DNA-binding proteins. Purified CTF or NF-I polypeptides were combined with end-labeled DNA fragments containing either control or CCAAT sequences, and the resulting protein–DNA complexes were incubated with polyclonal antibodies directed against the 60–66 kd polypeptide subgroup of NF-I (see Experimental Procedures). The DNA fragments that contain the α-globin CCAAT box were specifically recognized by either CTF or NF-I (Figure 4, lanes 4 and 6) and precipitated by the anti-NF-I antibodies. No bound fragments were detected in similar reactions in which pre-immune serum was substituted for immune serum (Figure 4, lanes 3 and 5) or in reactions lacking the DNA-binding protein (Figure 4, lane 7). Thus antibodies raised against a subfraction of the NF-I polypeptides efficiently recognize proteins present in both the CTF and NF-I preparations that interact specifically with the α-globin promoter.

Base-Substitution Mutants in the Adenovirus Origin and the HSV-*tk* Promoter

A region of 2-fold symmetry exists within the adenovirus origin sequence, TTGG(N)$_6$GCCAA, and sites that conserve this symmetry (such as the Ha-ras and origin sites) display a higher affinity for CTF and NF-I than sites that are not symmetrical (such as the human α-globin, β-globin, and HSV-*tk* promoters). To investigate the relative affinity of binding to apparent half-sites versus fully-symmetrical sites, point mutations in the adenovirus origin were constructed, and binding was analyzed with the DNAase I footprint method. Single-base substitutions in either domain of the symmetrical NF-I binding site were found to lower the affinity about 4-fold, and resulted in a

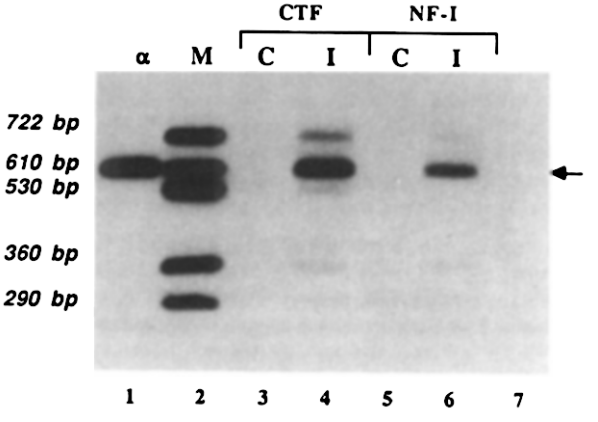

Figure 4. Promoter Fragment Immunoprecipitation Experiments

The radioactive DNA fragments used in this assay (M; lane 2) were a 722 bp fragment of the proximal promoter (−300 to +400) of the Drosophila alcohol dehydrogenase gene; a 610-bp fragment (−510 to +100) of the human alpha-1 globin gene (also shown in lane 1); a 530-bp fragment (−500 to +30) of the human ribosomal RNA promoter; and 360-bp (−340 to +20) and 290-bp (+20 to +270) fragments of the human immunodeficiency virus promoter. DNA fragments were incubated with CTF (Ha-Ras; 10 ng, lanes 3 and 4) or NF-I (10 ng; lanes 5 and 6), and immunoprecipitated, as described in Experimental Procedures, with a polyclonal antibody serum (I; lanes 4, 6, and 7) directed against the 60K–66 kd polypeptide group of NF-I, or with pre-immune serum (C; lanes 3 and 5). A control reaction containing labeled DNA, polyclonel antibodies, and S. aureus cells, but lacking CTF or NF-I, was run on lane 7. The samples were electrophoresed through a 2% agarose gel, and visualized by autoradiography with an intensifying screen for 2 hr at −80°C.

protected region approximately 8 bp smaller than the wild-type footprint (Figure 5A). Because elements of symmetry still remain in the NF-I point mutants, these values must be considered as the minimal differences in affinity between the two types of binding sites. These data, along with the observed binding of CTF/NF-I to CCAAT boxes in various promoters, strongly suggest that the minimal recognition site for CTF/NF-I is a single AGCCAA motif, which, when present as an inverted repeat with a center-to-center distance of 10 bp (approximately one turn of the DNA helix), generates an exceptionally high-affinity binding site.

Previous studies established a positive correlation be-

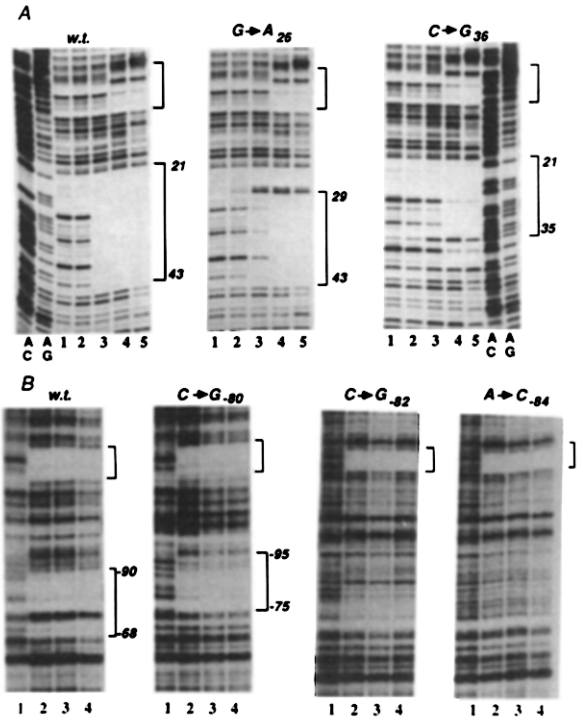

Figure 5. Interaction of CTF and NF-I with Single-Base Substitution Mutants

(A) Binding of NF-I to point mutants in the adenovirus origin of DNA replication. Plasmids pUpm26 and pUpm36 were 3′ end labeled at the BamHI site and incubated with the following amounts of affinity-purified NF-I: 0 ng, 0.1 ng, 0.4 ng, 1.6 ng, 6.4 ng; lanes 1 through 5, respectively.

(B) Binding of CTF to point mutants in the HSV thymidine kinase promoter. The *tk* promoter mutants pm −80, pm −82, and pm −84 (Graves et al., 1986) were 5′ end labeled at the BglII site and incubated with the following amounts of affinity-purified CTF (α-globin): 0 ng, 15 ng, 20 ng, 30 ng; lanes 1 through 4, respectively.

tween the binding of CTF and transcriptional activation of the HSV-*tk* promoter in vitro (Jones et al., 1985). We have extended this correlation by analyzing the transcriptional properties and the binding of affinity-purified CTF to point mutants (Graves et al., 1986) in the *tk* promoter CCAAT box. Two of the base substitutions (pm −82 and pm −84) eliminate the binding of CTF, whereas a third mutant (pm −80) did not alter the binding affinity in DNAase footprint experiments (Figure 5B). Significantly, the transcriptional properties of these mutants in vivo (Graves et al., 1986) and in vitro correlate with the ability to bind CTF; that is, mutants pm −82 and pm −84 are transcribed at levels approximately 10% that of the wild-type promoter, whereas mutant pm −80 is expressed at 140% of wild type in vivo and 120% of wild type in vitro. In addition, these results confirm that CTF recognizes the half-site sequence, CGCCAAT, in the HSV-*tk* promoter.

In Vitro Stimulation of Replication and Transcription by CTF and NF-I

Because our results indicate that CTF and NF-I contain the same set of DNA-binding polypeptides, it was important to compare the biochemical activities of each protein fraction with in vitro reactions that measure transcription and DNA replication. The initial steps in adenovirus replication involve covalent attachment of the viral precursor terminal protein (pTP) to the first deoxynucleotide residue (a cytidine-5′-monophosphate) of the new DNA strand. The formation in vitro of the pTP-dCMP initiation complex is dependent on the adenovirus DNA polymerase, the viral origin sequences, and several nuclear proteins that interact specifically with the origin, including NF-I (Rosenfeld et al., 1987). In reactions containing purified viral replication proteins and the adenovirus replication template, a basal level of pTP-dCMP complex formation is observed (Figure 6A). The initiation reaction is strongly stimulated by the addition of a partially purified HeLa nuclear fraction (BR-FT), which is depleted of NF-I activity and includes at least two other proteins that bind specifically to the adenovirus origin (Rosenfeld et al., 1987). The addition of affinity-purified CTF or NF-I to reactions containing the template, viral proteins, and the BR-FT fraction resulted in a 10-fold increase in pTP-dCMP levels. Interestingly, CTF and NF-I did not appreciably stimulate the reaction in the absence of the BR-FT fraction. Thus the two fractions behaved in an identical manner in the replication assay in that each stimulated pTP-dCMP complex formation only in the presence of the BR-FT fraction.

The ability of CTF and NF-I to activate RNA synthesis from the human α-1 globin promoter was tested with in vitro transcription reactions. Whole-cell HeLa extracts were used to prepare a reconstituted transcription system (Dynan and Tjian, 1983) that contains the endogenous RNA polymerase II activity, as well as other general initiation factors, and is largely devoid of CTF activity. RNA synthesized in the presence or absence of CTF and NF-I was measured by primer extension reactions using a synthetic oligodeoxynucleotide complementary to the α-globin gene (Figure 6B). Reactions that contained only RNA polymerase II and the general transcription factors generated a relatively low level of accurately initiated RNA from the wild-type α-globin promoter template. The addition of affinity-purified CTF or NF-I to these reactions resulted in a 5-fold activation of RNA synthesis from either the wild-type promoter or a mutant α-globin template that lacked sequences upstream of the CTF-binding site (dl-87). In contrast, CTF-dependent transcription was not observed with a deletion mutant (dl-55) that removes the CTF-binding site but retains the TATA homology (Figure 6B). Transcription from an internal control template, the adenovirus major late promoter, was not affected by either CTF or NF-I. Moreover, the BR-FT fraction that stimulates adenovirus DNA replication does not influence RNA synthesis from the α-globin promoter (data not shown).

Discussion

The CCAAT-binding transcription factor, CTF, was purified by sequence-specific DNA affinity chromatography and consists of a family of polypeptides of molecular weight range similar to the adenovirus DNA replication stimulatory factor, NF-I (Rosenfeld and Kelly, 1986). Independently purified CTF and NF-I fractions were indistin-

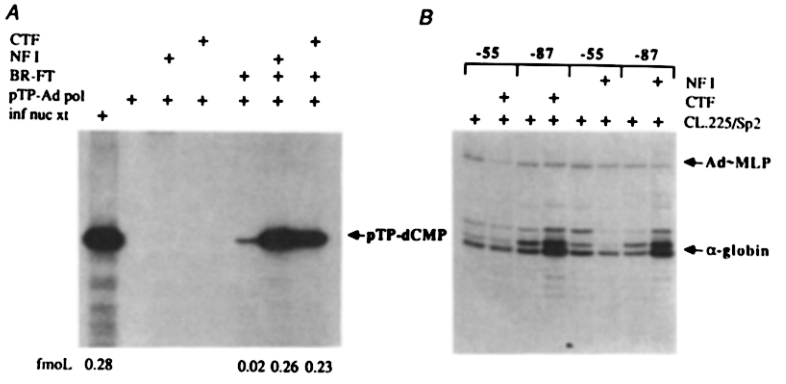

Figure 6. CTF/NF-I Stimulates In Vitro pTP-dCMP Complex Formation and Transcription Initiation from the Human α-Globin Promoter

(A) Stimulation of pTP–dCMP complex formation by CTF and NF-I. Formation of the pTP-dCMP complex was assayed as described in Experimental Procedures. Nuclear extract from adenovirus-infected HeLa cells served as a control for the initiation reaction. The viral proteins (pTP-Ad pol; 0.6 μg; 0.004 polymerase units), prepared as described by Rosenfeld et al. (1986), were added in the absence or presence of additional protein fractions required for the initiation of adenovirus DNA replication in vitro. Reactions further contained: BR-FT (7.5 μg), a cellular fraction required for optimal initiation activity (Rosenfeld et al., 1987); and affinity-purified NF-I (10 ng; 75 units) or CTF (α-globin; 75 U) as indicated. One unit of binding activity is defined as the amount of protein required to bind 1 fmol of the NF-I recognition sequence in the adenovirus origin of DNA replication.

(B) Stimulation of transcription in vitro from the human α-1 globin promoter by CTF and NF-I. RNA synthesized in vitro from 5′-deletion α-globin templates that lack (−55) or carry (−87) the CTF-binding site was measured by primer extension. Each reaction contained 150 ng of adenovirus DNA template, 2 ng of adenovirus major late promoter template (Ad~MLP), 36 μg of the CL.225 fraction, 2.5 μg of the Sp2 fraction, and 10 ng of affinity-purified CTF (Ha-ras) or NF-I, as indicated. Arrows indicate accurately initiated RNA transcripts.

guishable by direct comparison of polypeptide composition and biological activities. We conclude that CTF/NF-I stimulates RNA polymerase II transcription and the formation of adenovirus DNA replication initiation complexes through binding to DNA control elements that contain the sequence motif GCCAAT. Therefore, a cellular factor clearly implicated in transcription is used by adenovirus to stimulate the formation of the initiation of viral DNA replication. Whether CTF/NF-I also plays a role in cellular DNA replication remains to be determined.

Both purified CTF and purified NF-I contain a heterogeneous population of polypeptides in the molecular weight range 52–66 kd. Partial proteolysis experiments carried out by Rosenfeld and Kelly (1986) indicated that these polypeptides are related to each other in primary structure. Thus the observed heterogeneity is probably not due to contamination with unrelated proteins. It is possible that the different polypeptides are generated by posttranslational modification or are encoded by different (but related) genes. It is also possible that they are generated by proteolytic degradation of a larger CTF/NF-I protein species. Diffley and Stillman (1986) have reported the identification of a 160K polypeptide in highly purified preparations of NF-I. We have attempted to confirm this report by repeating the purification of CTF/NF-I in the presence of the protease inhibitors used by Diffley and Stillman, but we have not observed a species of higher molecular weight. In addition, Western blot analysis of crude HeLa nuclear extracts with polyclonal antibody raised against NF-I has revealed only the 52–66 kd CTF/NF-I polypeptides (P. R. and T. K., unpublished data; C. Santoro and R. Tjian, unpublished data).

The competition experiments reported here eliminate the possibility that CTF/NF-I is a complex of proteins that has a separate and independent adenovirus origin and CCAAT-binding domains. The formal possibility exists, however, that different sets of polypeptides (which bind identically to DNA) are responsible for the transcription and replication activities. It is also possible that only a subset of the proteins that bind to DNA are competent to activate transcription or replication. Thus it will be important to understand the nature of CTF/NF-I heterogeneity and to determine the protein domains that are responsible for the transcription and replication stimulation activities.

Given the detailed information available on the binding of NF-I to DNA, how is it that CTF and NF-I were not previously recognized to be the same protein? Part of the answer lies in the complexity of the CTF/NF-I recognition site. Affinity-purified CTF/NF-I recognizes CCAAT-related sequences with affinities that vary according to one, the extent to which the recognition sequence matches the consensus (AGCCAA), and two, whether or not the consensus is present in a 2-fold rotationally symmetric manner. For example, the symmetrical sequence TTGGCT-$(N)_3$AGCCAA is predicted to be one of the highest-affinity CTF/NF-I sites. The adenovirus origin site, TTGGAT$(N)_3$-AGCCAA, differs from a perfectly symmetrical site by only one nucleotide and has an exceptionally high binding constant ($K_D \sim 10^{-11}$ M; 150 mM NaCl, 22°C). Mutation of this site to the perfectly symmetrical sequence increases the NF-I binding affinity 4-fold (Rosenfeld et al., 1987). Similarly, point mutations in either half of the adenovirus origin binding site reduce the binding affinity by a factor of four (Figure 5A) to a value comparable to the affinity for the α-globin promoter. These results, together with the observation that most of the promoter CTF sites lack notable symmetry (Figure 7), suggest that CTF/NF-I can bind with high affinity to single AGCCAA sequences. It is important

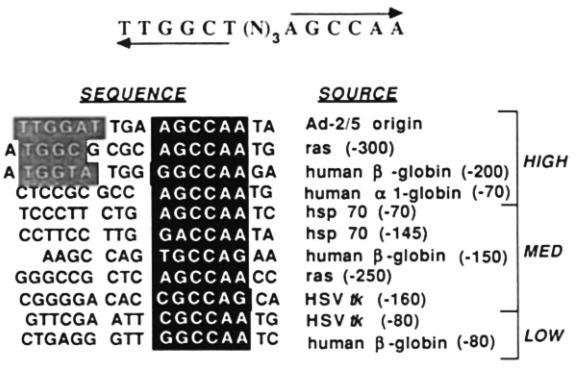

Figure 7. Sequence Comparison of CTF/NF-I Binding Sites

Sequences of some of the promoter binding sites mentioned in the text were aligned according to the best fit to the motif AGCCAAT and grouped by relative affinity for CTF, as approximated by DNAase I footprint analysis. High, medium, and low affinities are distinctive by a factor of at least five.

to note, however, that the nucleotide contacts of CTF/NF-I have been established only for the adenovirus origin and the HSV-*tk* binding sites, and that contact residues of the other sites listed in Figure 7 have not been determined. It is possible, for example, that CTF/NF-I interacts with the sequence CGCCAG, rather than the sequence AGCCAA, in the α-globin binding site. Detailed binding studies on one or more of the single AGCCAA sequences will therefore be required to establish with certainty each of the major nucleotide determinants for CTF binding.

There exists a large body of evidence that links the CCAAT motif to transcriptional activation. Specific CTF elements have been found in the Xenopus and human *hsp70* promoters, in addition to the *tk* and vertebrate globin genes. Mutations that modify the CCAAT elements of the HSV-*tk*, β-globin, and Xenopus *hsp70* genes reduce RNA synthesis as much as 10-fold in vivo (McKnight et al., 1984; Graves et al., 1986; Charnay et al., 1985; Myers et al., 1986; Bienz and Pelham, 1986), and deletion of the α-globin CCAAT site reduces transcription in vivo at least 25-fold (Mellon et al., 1981). The HSV-*tk* and α-globin promoter mutants have less severe phenotypes in vitro, and CTF activation is correspondingly weaker. CTF activates the α-globin promoter in vitro only 5-fold, instead of 25-fold. The low in vitro transcriptional activity of CTF may be a consequence of modification and/or proteolysis of the factor, such that only a subset of those CTF proteins that bind to DNA are active for transcription. Alternatively, other factors that act synergistically with CTF may not be present at optimal levels in the cell-free transcription system. NF-I sites have also been found in the upstream regions of several other genes, including the mouse mammary tumor virus promoter, the chicken lysozyme gene, the human *c-myc* gene, and the BK virus enhancer (Borgmeyer et al., 1984; Nowock et al., 1985; Hennighausen et al., 1985). It is not clear, however, whether these potential NF-I binding sites actually contribute to the expression of these genes, or whether they play a role in cellular DNA replication. In this regard, it is important to note that the very high affinity CTF-binding sites located in the distal upstream region of the human β-globin promoter (−125 to −300 region; Figure 7) do not contribute to the expression of the β-globin gene in transient expression experiments, whereas the much lower affinity binding site located at position −80 is fully functional. Thus neither the mere presence of a CCAAT sequence in the vicinity of a promoter nor the binding of CTF to a fortuitous CCAAT element is sufficient per se to create a promoter responsive to CTF, but, rather, transcriptional activation is determined by the context of the binding site in relation to the location of other transcription factors.

Surprisingly, our results indicate that CTF is distinct from a rat liver protein, CBP, which interacts with a closely related overlapping sequence in the HSV-*tk* promoter (Graves et al., 1986). First, the chromatographic and heat-stability properties of the two activities are different, and the two fractions do not generate identical footprint patterns on the HSV1 *tk* promoter (Jones et al., 1985; Graves et al., 1986). The different footprint patterns are not due to species or cell-type distinctions because rat liver extracts contain CTF activity (K. J. and R. T., unpublished data). Second, the binding to the HSV-*tk* point mutants reported here indicates that the recognition elements of CBP and CTF are different. For example, a single-base substitution mutant (pm −82), which changes the CCAAT sequence to GCAAT and inhibits transcription in vitro and in vivo, eliminates the binding of CTF but enhances the binding of CBP. Thus the binding of CTF to the *tk* promoter correlates well with the transcriptional activity of the promoter mutants. That the *tk* promoter element is in fact a true CCAAT box is supported by the observation that affinity-purified CTF/NF-I also binds to canonical CCAAT elements in the human globin genes and activates α-globin transcription in vitro.

One final intriguing question is whether the mechanism by which CTF activates the formation of the pTP-dCMP replication initiation complex is related to that involved in the activation of RNA synthesis. For example, the binding of CTF may influence the local DNA structure of the promoter and origin regions in a manner that influences the recognition of replication or transcription components. Alternatively, CTF may interact directly with other factors by protein–protein contacts. Mutational analysis of CTF should determine whether one or more domains of the protein are responsible for these two distinct functions. Stimulation of pTP-dCMP complex formation also requires a cellular fraction, BR-FT, which contains other proteins that bind to the adenovirus origin of DNA replication (Rosenfeld et al., 1987). Recent studies indicate that one of these stimulatory proteins (ORP-C) binds specifically to the octamer element (ATGCAAAT) that is required for optimal transcription from a variety of cellular promoters (Falkner and Zachan, 1984; Parslow et al., 1984; Ephrussi et al., 1985; Mason et al., 1985; Sive and Roeder, 1986; Singh et al., 1986; Pruijn et al., 1986; E. A. O'Neill, P. J. Rosenfeld, and T. J. Kelly, unpublished data). Thus the observation that CTF/NF-I activates both viral DNA replication and transcription may reflect a more general phenom-

enon in which a given site-specific DNA-binding protein could participate in a variety of different cellular processes.

Experimental Procedures

Sequence-Specific DNA Affinity Chromatography

Resins were prepared according to Kadonaga and Tjian (1986), using the specific oligodeoxynucleotide sequences shown in Figure 1. A HeLa nuclear extract (32l) was fractionated by Sephacryl S-300 chromatography as described by Briggs et al. (1986); CTF activity was monitored by footprint reactions with the human α-1 globin and HSV-*tk* promoter fragments. CTF activity is purified approximately 5- to 10-fold by this gel-filtration step. The S-300 fraction (volume, 40 to 100 ml; protein concentration, approximately 0.5 mg/ml) was incubated for 10 min at 4°C with 8 μg/ml poly (dI-C) plus 2 μg/ml calf thymus DNA, and passed in parallel over six 1 ml oligodeoxynucleotide columns of the specific resin at 4°C. Columns were washed extensively with Buffer Z (25 mM HEPES, K^+; 12.5 mM $MgCl_2$; 1 mM dithiothreitol; 20% [v/v] glycerol; and 0.1% [v/v] Nonidet P-40) containing 100 mM KCl, and CTF was eluted with step gradients of Buffer Z containing 0.6 m KCl. First-pass eluates (volume 15–20 ml) were diluted to 0.1 m KCl with Buffer Z, incubated with 2 μg/ml poly d(I-C) (second pass) or 0.5 μg/μl poly d(I-C) (third and additional passes), and subjected to repeated chromatography over four 1 ml columns (second pass) or three 0.8 ml columns (third pass). The second and third pass fractions were purified 500-fold over the S-300 fraction (a total of 5000-fold over the nuclear extract) with an overall yield of approximately 30%–50% from the S-300 fraction. Aliquots (generally 5–10 μg/ml CTF) were frozen in liquid nitrogen and stored at −80°C. Protein fractions were precipitated as described by Kadonaga and Tjian (1986) and resuspended in 10 μl of sample buffer lacking 2-mercaptoethanol or dithiothreitol for analysis by SDS–polyacrylamide gel electrophoresis.

DNAase I Footprint Analysis

The plasmids used for footprint probe preparation were: pRB α-1, which consists of a 3 kb insert of the human α-1 globin gene into the PstI site of pBR 322; pHu (β), which contains a Pst fragment insert into pBR 322 of the human β-globin gene; HSV-*tk* promoter and CCAAT-box point mutants (McKnight, 1984; Graves et al., 1986); and plasmid pUdl 67, which contains a 67 bp fragment of the Ad2 genome inserted between the EcoRI and BamHI sites of the vector pUC 9. DNA plasmids were digested and the resulting 5′ termini were treated with calf intestinal alkaline phosphatase and 5′ end labeled with γ-^{32}P ATP (7000 Ci/mmol) and T4 polynucleotide kinase at the sites indicated in the individual figure legends. The radioactive DNA fragments were subsequently cleaved with an appropriate second enzyme, and fragments containing the binding sites were isolated by preparative polyacrylamide gel electrophoresis and electroelution. Varying amounts of CTF or NF-I were incubated with the radioactive DNA fragments (10 fmol per reaction) at room temperature for 10 min (promoter fragments) or 30 min (adenovirus origin fragment) in a 50 μl reaction containing 12.5 mM HEPES (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, and 0.5 mM dithiothreitol. DNAase I digestion and work-up procedures were as described (Jones et al., 1985).

Competition Experiments

Competition footprint experiments were carried out with ligated, double-stranded oligodeoxynucleotides containing the adenovirus NF-I site, the α-globin CCAAT element, or control oligodeoxynucleotides carrying binding-site sequences for the Sp1, AP-1, or AP-2 transcription factors. The average size of the ligated oligonucleotides was approximately 400 bp. The NF-I footprint was monitored with a 5′ end labeled HindIII–PvuII fragment of plasmid pUBS+, which contains the adenovirus DNA sequences (bp 19 to bp 46) in the polylinker region of pUC9. The α-globin footprint probe was the same as described above. Three nanograms of affinity-purified CTF was mixed with the appropriate competitor DNA and then added to each binding reaction. Binding reactions were incubated for 15 min at 0°C before DNAase I digestion. Competitor DNA concentrations are listed in moles of binding sites for the appropriate factor.

Gel Mobility Shift DNA-Binding Assay

The standard gel electrophoresis DNA-binding assay was a modification of previously published protocols (Fried and Crothers, 1981; Garner and Revzin, 1981; Singh et al., 1986). Plasmid DNAs were linearized with BamHI and labeled at their 3′ termini by incubating with α-^{32}P-dATP and α-^{32}P-dTTP in the presence of Micrococcus luteus polymerase. The radioactive DNAs were cleaved with PvuII and the appropriate fragments were isolated by electroelution after electrophoresis through an 8% polyacrylamide gel. The standard binding assay (25 μl) contained 25 mM HEPES (pH 7.5), 50 mM NaCl, 10% glycerol, 0.05% Nonidet P-40, 5 mg BSA, 1 mM DTT, 1 mM EDTA, 1 μg poly d(I-C) (Pharmacia P-L Biochemicals), and 0.1 nM ^{32}P-DNA fragment. The assays were incubated at 25°C for 30 min and electrophoresed at 10 V/cm through a 6% polyacrylamide gel (30% acrylamide; 0.8% bisacrylamide) that had been pre-electrophoresed for 2 hr at 10 V/cm. The gel was prepared with 12 mM Tris-acetate (pH 7.5), 1 mM EDTA, and 0.01% Nonidet P-40, and the gel buffer was recirculated during electrophoresis. After 90 min the gel was dried, and radioactivity was detected by exposure to Kodak XAR-5 film with an intesifying screen at −70°C.

Fragment Immunoprecipitation

Binding reactions were prepared that contained a mixture of 5′ end–labeled promoter fragments (0.7 fmol of each fragment per reaction) in a 25 μl volume containing 0.4% polyvinyl alcohol and 80 μg/ml poly d(I-C). An equal volume of binding buffer (Buffer Z) was added with 10 ng CTF or NF-I with final KCl concentrations adjusted to 50 mM, and the reactions were incubated for 10 min at room temperature. The reactions were subsequently diluted with 100 μl binding buffer, and 5 μl (50 μg) crude rabbit anti-NF-I polyclonal serum (or pre-immune serum) was added. The protein–DNA complexes were allowed to interact with the antibody for 20–30 min on ice. Formalin-fixed Staphylococcus aureus cells (8 μl per reaction; in binding buffer) were added and the reaction mixture was further incubated 10–15 min on ice. Antigen–protein–DNA complexes were precipitated by a 35 sec microfuge spin, and the pellets were resuspended with a siliconized glass rod in 200 μl of Buffer Z containing 200 mM KCl at room temperature. The pellets were then collected with a 1 min microfuge spin and resuspended well in a 10 μl dye-sucrose mixture containing 2% SDS. The S. aureus cells were removed by centrifugation, and the samples were loaded onto a 2% agarose gel alongside DNA markers of the input fragments, which had been resuspended in dye-sucrose without SDS to prevent streaking. The gel was subsequently dried and exposed to XAR-5 film for 2 hr at −70°C with an intensifying screen.

The antiserum was raised by initial lymph node injection of gel-purified NF-I (200 μg) into a white rabbit followed by subcutaneous boosts of approximately 100 μg of NF-I. Polypeptides in the 60K–66K molecular weight range had been excised from a preparative SDS–polyacrylamide gel of DNA affinity-purified NF-I stained with Coomassie blue. The S. aureus cells had been freshly thawed, sonicated for 1 min at 0°C in a cup sonicator, washed in Buffer Z containing 1 m $LiCl_2$, and then washed extensively in binding buffer before use.

Preterminal Protein-dCMP Complex Formation

The standard in vitro initiation reaction was performed as previously described (Rosenfeld and Kelly, 1986), with the following minor modifications: first, prior to incubation at 37°C, the initiation assays were adjusted to a final concentration of 30 mM NaCl and 10% glycerol; and second, the amount of radioactive label incorporated into the pTP-dCMP complex was determined after electrophoresis and autoradiography of the dried gel. The gel segment containing the initiation complex and an adjacent segment of identical size were excised, and the radioactivity was measured by liquid scintillation in Betafluor (National Diagnostics). The amount of pTP–dCMP complex formed was calculated from a standard curve prepared under identical conditions with known amounts of radioactive nucleoside triphosphates.

In Vitro Transcription Reactions

The general transcription fractions (CL .225 and Sp2) were prepared as described (Jones et al., 1985). Transcription reactions (50 μl) con-

tained 2 ng of the adenovirus major late promoter construct, pALP, 150 ng of α-globin template, 36 μg CL .225, 2.5 μg Sp2, and 125 ng CTF or NF-I. α-globin deletion mutants are described by Mellon et al. (1981). Transcription reactions were incubated for 45 min at 30°C and primer extension reactions were prepared as described (Jones et al., 1985). RNA from the adenovirus major late promoter was detected using a synthetic pBR primer (sequence: 5′-TCACCGTCATCACCGAAACGC-GCG-3′), and α-globin transcription was detected using a synthetic primer complementary to the globin gene (sequence: 5′-GGTTCTTGT-CGGCAGGAGACAGCACC-3′). The primers had been 5′ end labeled with ^{32}P using T4 polynucleotide kinase, and 25 fmol of each primer was used per reaction.

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