MOLECULAR AND CELLULAR BIOLOGY, Dec. 1986, p. 4723–4733
0270-7306/86/124723-11$02.00/0

Vol. 6, No. 12

A Single Polypeptide Possesses the Binding and Transcription Activities of the Adenovirus Major Late Transcription Factor

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Received 11 August 1986/Accepted 17 September 1986

A simple approach has been developed for the unambiguous identification and purification of sequence-specific DNA-binding proteins solely on the basis of their ability to bind selectively to their target sequences. Four independent methods were used to identify the promoter-specific RNA polymerase II transcription factor MLTF as a 46-kilodalton (kDa) polypeptide. First, a 46-kDa protein was specifically cross-linked by UV irradiation to a body-labeled DNA fragment containing the MLTF binding site. Second, MLTF sedimented through glycerol gradients at a rate corresponding to a protein of native molecular weight 45,000 to 50,000. Third, a 46-kDa protein was specifically retained on a biotin-streptavidin matrix only when the DNA fragment coupled to the matrix contained the MLTF binding site. Finally, proteins from the most highly purified fraction which were eluted and renatured from the 44- to 48-kDa region of a sodium dodecyl sulfate-polyacrylamide gel exhibited both binding and transcription-stimulatory activities. The DNA-binding activity was purified 100,000-fold by chromatography through three conventional columns plus a DNA affinity column. Purified MLTF was characterized with respect to the kinetic and thermodynamic properties of DNA binding. These parameters indicate a high degree of occupancy of MLTF binding sites in vivo.

The rate of transcription initiation is an important determinant of gene expression in eucaryotic organisms. Considerable effort has been devoted to elucidating the molecular mechanisms responsible for regulating the transcription initiation rate of specific genes. Genetic analysis of RNA polymerase II transcription units has defined at least three qualitatively different types of *cis*-acting DNA regulatory elements: the TATA box, upstream promoter elements, and transcriptional enhancers. Recently, the gel electrophoresis DNA-binding assay and the DNase footprinting assay have been used to demonstrate that each of these types of elements interacts with one or more sequence-specific DNA-binding proteins (4, 6, 21, 23). Furthermore, proteins which bind to the TATA box and to at least three distinct upstream promoter elements have been shown to stimulate transcription in vitro by RNA polymerase II (4, 5, 6, 21, 22, 32). One of these transcription factors, MLTF or USF, binds to and stimulates transcription from the major late promoter (MLP) of adenovirus (4, 18, 32). An understanding of the mechanisms by which MLTF and other transcription factors control transcription initiation requires the identification, purification, and characterization of these proteins. In addition, isolation of the genes encoding these proteins will permit study of their regulation during the course of cell growth and development and will allow genetic analysis of the structural characteristics which specify their transcriptional interactions.

The existence of MLTF was first suggested by the dependence in vivo of transcription from the adenovirus type 2 MLP on sequences located 50 to 66 base pairs (bp) upstream of the transcription initiation site (10, 13). DNase I footprinting and gel electrophoresis DNA-binding assays were subsequently used to identify a protein in uninfected cell extracts which recognized the 17-bp sequence between −50 and −66 (4, 18, 32). Although MLTF was not purified to homogeneity, its sequence-specific DNA-binding activity cofractionated with an activity that stimulated transcription in vitro from templates containing the MLTF binding site (4, 32). On this basis, it was suggested that efficient transcription from the MLP in vivo required the binding of MLTF to an upstream element.

We have developed a rapid approach for the identification and purification of sequence-specific DNA-binding proteins. First, the molecular weight of the DNA-binding protein was determined by photochemical cross-linking to a body-labeled DNA fragment containing its binding site. Second, the DNA-binding protein was specifically purified by its selective adsorption onto a streptavidin-agarose matrix via a biotinylated DNA fragment containing its binding site. Using these techniques, we have identified MLTF as a 46-kilodalton (kDa) polypeptide, purified it over 100,000-fold, and demonstrated that a single polypeptide is responsible for both the binding and transcription-stimulatory activities. Moreover, we have analyzed the kinetic and thermodynamic properties of DNA binding by the purified MLTF protein.

MATERIALS AND METHODS

Gel electrophoresis DNA-binding assay. Binding reactions and gel electrophoresis were all performed as described by Carthew et al. (4). DNA probes and competitors containing the adenovirus type 2 major late promoter were prepared from plasmids pLP and pRW. To construct pLP, the plasmid pFLBH (29) was digested with *Pvu*II and *Dde*I and blunted with Klenow fragment, and the 207-bp fragment (−174 to +33 relative to the major late promoter start site) was cloned into the *Sma*I site of pUC13 by standard cloning procedures. To construct pRW, the plasmid pFLBH was digested with *Pvu*II and *Nci*I and blunted with S1 nuclease, and the 84-bp fragment (−51 to +33 relative to the major late promoter start site) was cloned into the *Sma*I site of pUC13. To prepare DNA probes ML+ and ML−, pLP and pRW, respectively, were digested with *Eco*RI and *Hin*dIII and end-labeled with Klenow fragment and [α-^{32}P]dATP. The 256-bp fragment derived from pLP (designated ML+) and

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the 133-bp fragment derived from pRW (ML−) were purified by agarose gel electrophoresis. Competitor DNA fragments were prepared from pLP or pRW as described for probe preparation, except that DNA was not end-labeled. Other competitor DNA fragments were prepared as described by Carthew et al. (4).

In vitro transcription. Preparation of transcriptional fractions from HeLa cells, in vitro synthesis of RNA, and gel electrophoresis of RNA products were all as described by Samuels et al. (29). DNA templates were prepared and used as described by Carthew et al. (4).

Purification of MLTF. (i) Phosphocellulose and DEAE-Sephacel chromatography. HeLa whole-cell extract was prepared and chromatographed through phosphocellulose and DEAE-Sephacel columns as described by Samuels et al. (29). The chromatography buffer was buffer A (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–NaOH (pH 7.9), 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol [DTT]). Salt elutions from columns were performed with KCl. As described by Carthew et al. (4), MLTF-containing fractions eluted in the flowthroughs of the phosphocellulose and DEAE-Sephacel columns.

(ii) Heparin-Sepharose chromatography. The MLTF-containing flowthrough fraction (400 ml in buffer A + 0.15 M KCl) from the DEAE-Sephacel column was loaded onto a 3-ml heparin-Sepharose column (Pharmacia, Inc.) preequilibrated with buffer A + 0.15 KCl at a flow rate of 3 column volumes per h. After the column had been washed with 4 column volumes of buffer A + 0.15 M KCl and 5 column volumes of buffer A + 0.25 M KCl, proteins were eluted with a 15-ml linear gradient of KCl (from 0.25 to 0.6 M) in buffer A, and 0.3-ml fractions were collected. The concentration of KCl in the fractions was determined by conductivity. The MLTF-containing fractions 10 through 20 were pooled and dialyzed against 1 liter of buffer A + 0.1 M KCl for 4 h, and aliquots were stored at −70°C.

(iii) DNA affinity chromatography. DNA chromatography reagents were prepared and stored at 4°C for up to 2 months before use. DNA fragments were prepared by digestion of pLP or pRW with *Hin*cII and *Eco*RI. The *Eco*RI ends were filled in by incubation with Klenow fragment, 50 μM dATP, and 20 μM biotin-11-dUTP (Bethesda Research Laboratories) for 30 min at 23°C. The 239-bp fragment derived from pLP (−174 to +33 relative to the MLP start site) and the 116-bp fragment derived from pRW (−53 to +33 relative to the MLP start site) were purified by agarose gel electrophoresis, extracted twice with phenol-chloroform, and ethanol precipitated. The biotinylated fragments derived from pLP and pRW were designated BML+ and BML−, respectively.

Streptavidin-conjugated agarose beads (Bethesda Research Laboratories) were incubated with 3 volumes of buffer A with 60 mM KCl, 0.25 mg of bovine insulin (Sigma Chemical Co.) or bovine serum albumin (BSA) (Sigma) per ml, and 50 μg of poly(dI-dC) · (dI-dC) per ml for 30 min at 4°C. Beads were collected by centrifugation and immediately used for affinity adsorption.

The MLTF-containing fraction from the heparin-Sepharose column (20 μg of protein) was incubated with 2.0 pmol of either biotinylated BML+ or BML− in the presence of 1 or 0.1 mg of bovine insulin or BSA per ml and 0.1 mg of poly(dI-dC) · (dI-dC) per ml in a final volume of 0.2 ml. Incubations were performed at 30°C for 30 min in binding buffer (12 mM HEPES-NaOH [pH 7.9], 12% glycerol, 60 mM KCl, 5 mM $MgCl_2$, 4 mM Tris hydrochloride, 0.6 mM EDTA, 0.6 mM DTT). The binding reaction mixture was then incubated under constant rotation with 0.1 ml (packed volume) of pretreated streptavidin-agarose beads at 4°C for 16 h. The suspension was briefly centrifuged, and the supernatant was removed. The pellet was washed five times with 10 ml of binding buffer and poured into a column. The column was washed with 0.5 ml of buffer B (10 mM Tris hydrochloride [pH 6.8], 20% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.1% Nonidet P-40, 0.25 mg of bovine insulin or BSA per ml) + 0.15 M KCl, and 15-μl fractions were collected. The column was then washed with 0.5 ml of buffer B + 0.5 M KCl, and 15-μl fractions were collected. Fractions were assayed for DNA-binding activity. Peak fractions were pooled and stored in small aliquots at −70°C.

Analysis of proteins. The protein concentration was measured by the method of Bradford (1). Proteins were electrophoresed on discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gels (10 to 12.5% separating gel) as described by Samuels and Sharp (31). Silver staining after electrophoresis was carried out essentially as described by Morrissey (19), except that gels were first soaked in 40% methanol–10% acetic acid followed by subsequent changes in 10% ethanol–5% acetic acid and finally in 10% ethanol.

Glycerol gradient sedimentation analysis. A 100-μl portion of the MLTF-containing heparin-Sepharose fraction was diluted with 250 μl of 20 mM HEPES-NaOH (pH 7.9)–1 mM EDTA–1 mM DTT–100 mM KCl–7.5% glycerol. This was sedimented through a 4.8-ml 10 to 30% glycerol gradient in 20 mM HEPES-NaOH (pH 7.9)–1 mM EDTA–1 mM DTT–100 mM KCl at 45,000 rpm for 38 h at 4°C in a Beckman SW50.1 rotor. Fractions of 150 μl were collected from the top of the tube.

UV cross-linking. Body-labeled DNA from cross-linking was prepared by strand synthesis of an M13 recombinant bacteriophage. Single-stranded M13 XH10 (30), which contained nucleotides −261 to +197 of the major late promoter was hybridized to the 17-nucleotide universal primer (Pharmacia) and used as template for Klenow fragment in the presence of 50 μM dATP, dGTP, 5-bromo-2'-deoxyuridine triphosphate (Pharmacia), and 5 μM [α-^{32}P]dCTP. The DNA was digested with *Pvu*II and *Eco*RI, and the 322-nucleotide fragment (−261 to +33 relative to the MLP start site) was purified by agarose gel electrophoresis.

Protein samples (60 μg protein) from the DEAE-Sephacel flowthrough fraction were incubated with 1 ng of body-labeled DNA and 5 μg of poly(dI-dC) · (dI-dC) as above in 50 μl of binding buffer at 23°C. The mixture (in a Nunc vial sealed with Saran Wrap) was irradiated under a Fotodyne UV lamp (maximum emission wavelength, 310 nm; maximum intensity, 7,000 μW/cm^2) at a distance of 4.5 cm from the UV source. The mixture was brought up to 10 mM $CaCl_2$, and DNA was digested for 30 min at 37°C with 3.3 μg of DNase I (Worthington Diagnostics) and 1 U of micrococcal nuclease (Worthington). The mixture was then electrophoresed on a SDS-12.5% polyacrylamide gel as described above, and the gel was enhanced for fluorography, dried, and autoradiographed.

Renaturation of gel-purified MLTF. MLTF was electrophoresed, eluted, and renatured by the protocol of Hager and Burgess (9). MLTF fractions were electrophoresed on SDS-polyacrylamide gels (8.75% lower gel as described by Laemmli [14]), and 6- to 10-mm gel slices spanning a 18- to 200-kDa size range were cut out and eluted overnight at 23°C. The gel eluate was acetone precipitated, and the pellets were washed once with ice-cold 80% acetone. The dried pellets were dissolved in 6 M guanidine hydrochloride and incubated for 20 min at 23°C before dilution to 0.12 M guanidine hydrochloride. The protein was allowed to

renature overnight at 23°C and was tested for DNA binding and transcription-stimulatory activitiy.

Heat treatment of purified MLTF. Affinity-purified MLTF (M4) (6 μl) was incubated in the presence or absence of 5 mM $MgCl_2$ at 30, 40, 50, 60, 70, or 100°C for 5 min. Samples were placed on ice for 10 min and then incubated at 30°C for 10 min with 4 μl of a solution containing 0.4 ng of probe, yielding final concentrations of 5 mM $MgCl_2$, 4 mM Tris hydrochloride (pH 7.9), 0.6 mM EDTA, 12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, and 0.6 mM DTT. Gel electrophoresis and quantitation by densitometry were as described by Carthew et al. (4).

Quantitative equilibrium binding measurements. Binding of MLTF to ^{32}P-labeled DNA fragments was determined by the gel electrophoresis assay. Complexes were formed at increasing concentrations of MLTF, establishing the equilibrium $P + A \rightleftharpoons PA$, in which P is free DNA, A is free MLTF, and PA is protein DNA complex (assuming a 1:1 binding stoichiometry). If total DNA is in vast molar excess over total MLTF in the reaction, it may be shown that

$$[PA] = \frac{K_{eq}[P_T]}{1 + K_{eq}[P_T]} \cdot [A_T]$$

where $[P_T]$ and $[A_T]$ are total DNA and MLTF concentrations, respectively, and K_{eq} is the apparent equilibrium binding constant. The concentration $[A_T]$ was determined by the method of Riggs et al. (28), by measuring the concentration of protein-DNA complex when all of the MLTF was saturated with DNA. The concentration of complex gives the concentration of active MLTF. Active MLTF was then titrated against a 100-fold molar excess of DNA. The slope of the curve obtained by plotting complex concentration against total MLTF concentration was then used with the known $[P_T]$ to calculate K_{eq}.

In a typical binding experiment (with 20 μl), MLTF was diluted to a final concentration of 10^{-12} M and mixed with 1.8×10^{-10} M (final concentration) ^{32}P-labeled ML+ fragment in binding buffer plus 0.25 mg of BSA per ml. After 30 min at 30°C, the mixture was electrophoresed on a low-salt gel. The dried gel was autoradiographed at −70°C by using preflashed Kodak XAR film (15). The developed films were scanned with an LKB soft-laser scanning densitometer, and peak areas were integrated with an LKB 2220 integrator. Serial dilutions of input ^{32}P-labeled DNA were electrophoresed on the same gel. The signals from the DNA dilutions as measured by this method confirmed that signal was linearly proportional to input radioactivity in the DNA down to 0 cpm. The DNA dilutions also provided an internal standard with which to directly measure the bound DNA concentration.

Kinetic binding experiments. Dissociation rate constants (k_d) for MLTF-DNA complexes were measured by the gel electrophoresis technique of Fried and Crothers (7). MLTF (3×10^{-12} M) was incubated with 1.8×10^{-10} M ^{32}P-labeled ML+ DNA for 20 min at 30°C in binding buffer plus 0.25 mg of BSA per ml. This incubation was sufficient to attain binding equilibrium. The binding mixture was then incubated at 0 or 30°C. Dissociation measurements were initiated by addition of a 60-fold molar excess of unlabeled ML+. Aliquots were taken at the indicated times, chilled on ice, and electrophoresed on a low-salt gel at 4°C. Data were plotted according to the first-order rate equation given by Riggs et al. (27), and k_d was calculated by linear regression. The observed rates of dissociation were independent of the amount of unlabeled ML+ used as competitor.

Association rate constants (k_a) were measured by the gel electrophoresis assay. MLTF (0.2 to 2×10^{-12} M) was incubated with 3.6×10^{-10} M ^{32}P-labeled ML+ DNA in binding buffer plus 0.25 mg of BSA per ml at 0 or 30°C for various times. For reactions at 0°C, aliquots were taken at the indicated times and loaded onto a running low-salt gel. For reactions at 30°C, aliquots were taken, mixed with 60-fold excess unlabeled ML+ DNA to quench the association, chilled to 0°C to prevent significant dissociation, and electrophoresed on a low-salt gel at 4°C. Complex concentration was determined as described above, and data were plotted according to the linear transformation given by Riggs et al. (27). Since total DNA was in vast excess over total MLTF in the reaction mixture, the integrated rate equation simplified to $1/[P_T] \ln ([A] - [PA])/[A] = k_a t$. The association constant k_a was calculated by linear regression.

RESULTS

We previously identified a cellular protein, MLTF, which binds specifically and with high affinity to an upstream sequence element in the adenovirus MLP. The binding of this protein to a labeled DNA fragment in a soluble cell extract was detected by resolving nucleoprotein complexes from free DNA by nondenaturing gel electrophoresis (4). Having established conditions which maximize specific binding of MLTF to its target sequence while minimizing nonspecific binding of proteins to the probe, we used these conditions to devise procedures to identify and purify this protein.

Identification of MLTF by UV cross-linking. A photochemical cross-linking protocol was designed to selectively label MLTF on the basis of its specific interaction with the MLP. A DNA probe containing the MLTF binding site was prepared by incorporating bromodeoxyuridine and radioactive deoxycytidine into the noncoding strand (16). Substitution of bromodeoxyuridine for thymidine did not affect the binding of MLTF to the probe as assayed by gel electrophoresis of protein-DNA complexes (data not shown). A chromatographic fraction containing MLTF (DEAE-Sephacel fraction; Table 1) was incubated with the body-labeled probe under conditions identical to those routinely used in the gel electrophoresis DNA-binding assay. After the binding reaction had reached equilibrium, samples were irradiated with UV light and digested with DNase I and micrococcal nuclease. The molecular weights of the cross-linked proteins were determined by electrophoresis on SDS-polyacrylamide gels. The extensive nuclease digestion ensured that only short oligonucleotides from the probe DNA remained covalently cross-linked to proteins during electrophoresis. When probe DNA alone was irradiated (Fig. 1A, lane 1) or when UV irradiation was omitted (lane 2), no nuclease-resistant radioactively labeled species were generated. However, when complete binding reaction mixtures were irradiated and subsequently analyzed, several labeled species were generated. As expected, the amount of label in these species increased with increasing times of irradiation (lanes 3 through 6). The most prominent labeled species, which migrated as a protein of molecular weight 46,000, was visible after only 5 min of UV irradiation (lane 3). Control reactions demonstrated that probe DNA UV irradiated for 60 min was still able to bind MLTF (data not shown). Similarly, UV-irradiation of MLTF alone did not significantly reduce its ability to specifically bind probe DNA (data not shown). When UV-irradiated binding reaction mixtures were treated with proteinase K, no labeled species were observed follow-

TABLE 1. Purification of MLTF

Fraction	Total protein (mg)	Activity (pmol bound)	% Yield	Sp act (pmol bound/ mg of protein)	Purification (fold)
HeLa whole-cell extract	3,000	670		0.2	
A (phosphocellulose)	1,500	330	50	0.2	1.0
AA (DEAE-Sephacel)	670	210	32	0.3	1.4
M3 (heparin-Sepharose)	2	210	32	100	470
M4 (DNA affinity)	0.003	70	11	22,600	105,000

ing electrophoresis, suggesting that the labeled species were protein-DNA adducts (data not shown). Thus, UV irradiation predominantly resulted in the covalent attachment of MLP promoter DNA to one polypeptide.

The above experiments did not differentiate between polypeptides bound specifically and those bound nonspecifically to the DNA probe. To distinguish between these possibilities, UV cross-linking was performed in the presence of an excess of unlabeled competitor DNA which either contained or lacked the MLTF binding site. If a labeled polypeptide represents covalent cross-linking of MLTF to its target sequence, then competitor fragments containing this sequence should abolish formation of the corresponding labeled DNA-protein adduct. Conversely, competitor fragments lacking the MLTF binding site should not affect formation of the labeled DNA-protein adduct. Indeed, competitor fragments containing the MLTF binding site specifically abolished labeling of the 46-kDa polypeptide (Fig. 1B, lanes 5, 6, and 8). Longer autoradiographic exposures of this same gel showed that the specific competitor fragments had no effect on the intensity of the other photolabeled species (data not shown). Nonspecific competitor fragments which did not contain the MLTF binding site did not affect the degree of photolabeling of the 46-kDa polypeptide (lanes 7 and 9). Treatment of irradiated binding mixtures with either greater amounts of nuclease or the same amount of nuclease but for longer times did not alter the mobility of the 46-kDa species. Since the covalent attachment of short oligonucleotides to proteins has only a minor effect on the mobility of these proteins in SDS-polyacrylamide gel electrophoresis (11), these experiments indicate that a protein of approximately 46 kDa binds specifically to the MLTF-binding site.

Purification of MLTF by heparin-Sepharose chromatography. As previously described, MLTF binding activity flows through a phosphocellulose column when loaded at 40 mM KCl (fraction A) and also flows through a DEAE-Sephacel column when loaded at 150 mM KCl (fraction AA) (4). The latter fraction (AA) was loaded onto a heparin-Sepharose column at 150 mM KCl. MLTF bound to the heparin-Sepharose column at this salt concentration remained bound during a 0.25 M KCl wash and was eluted from the column on a linear KCl gradient at approximately 0.4 M KCl (Fig. 2A). Since the protein concentration in the peak fractions represented less than 0.3% of the total protein loaded onto the column (Fig. 2B, compare lanes Load and FT) and since 100% of MLTF binding activity was recovered from the column, this chromatographic step resulted in a >300-fold purification (Table 1).

Sedimentation analysis of MLTF activity. To estimate the native molecular weight of MLTF, material from the peak fractions of the heparin-Sepharose column was sedimented through a glycerol gradient. Following centrifugation, a portion of each gradient fraction was assayed for MLTF binding activity. Binding activity peaked in fraction 20, corresponding to a Svedberg coefficient of 3.6S (Fig. 3). For a globular protein with an average partial specific volume and degree of hydration, and S value of 3.6 S corresponds to a molecular weight of approximately 45,000 to 50,000 (3). Therefore, this sedimentation analysis is consistent with the molecular weight determination of 46,000 made on the basis of photochemical cross-linking.

Identification and purification of MLTF by affinity chromatography. We have devised a DNA affinity procedure by which sequence-specific DNA-binding proteins can be rapidly purified. In this method, the basis of which is illustrated in Fig. 4A, the same DNA fragment used for the gel electrophoresis DNA-binding assay was modified by incorporation of biotinylated deoxynucleotides into one end of the fragment. Low-specific-activity [α-^{32}P]dATP was also incorporated so that binding reactions could be conveniently monitored. To identify MLTF, two biotinylated DNA fragments were prepared, one from the wild-type MLP contain-

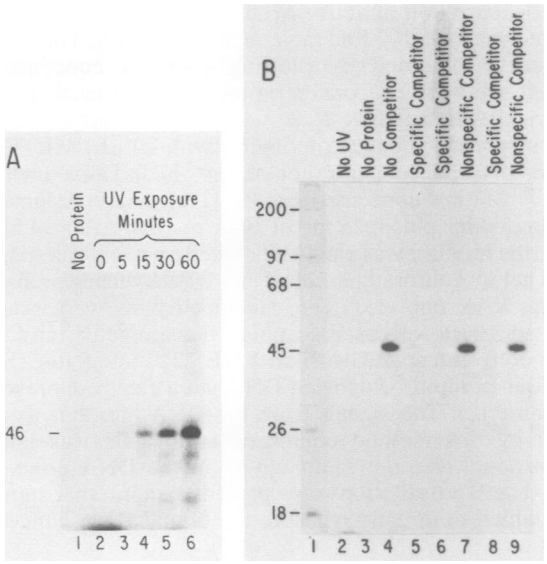

FIG. 1. Identification of MLTF by UV cross-linking. (A) Time course of cross-linking. Binding reactions containing body-labeled probe alone (lane 1) or probe plus 60 μg of protein from fraction AA (lanes 2 to 6) were UV irradiated for the indicated times and processed as described in Materials and Methods. (B) Specificity of cross-linking. Binding reactions (lanes 2 to 9) contained body-labeled probe alone (lane 3) or probe plus 60 μg of protein from fraction AA and an 80-fold molar excess of unlabeled DNA either containing or lacking the MLTF binding site (specific or nonspecific). Reactions were UV irradiated for 60 min and processed as above. Lanes: 1, protein molecular weight markers; 2, UV irradiation omitted; 3, probe alone; 4, no competitor; 5, MLP-containing fragment ML+; 6, MLP-containing fragment MLP_c (4); 7, MLTF$^-$ mutant major late promoter fragment ML−; 8, MLP-containing fragment MLP_a (4); 9, adenovirus type 5 pIX promoter-containing fragment (4).

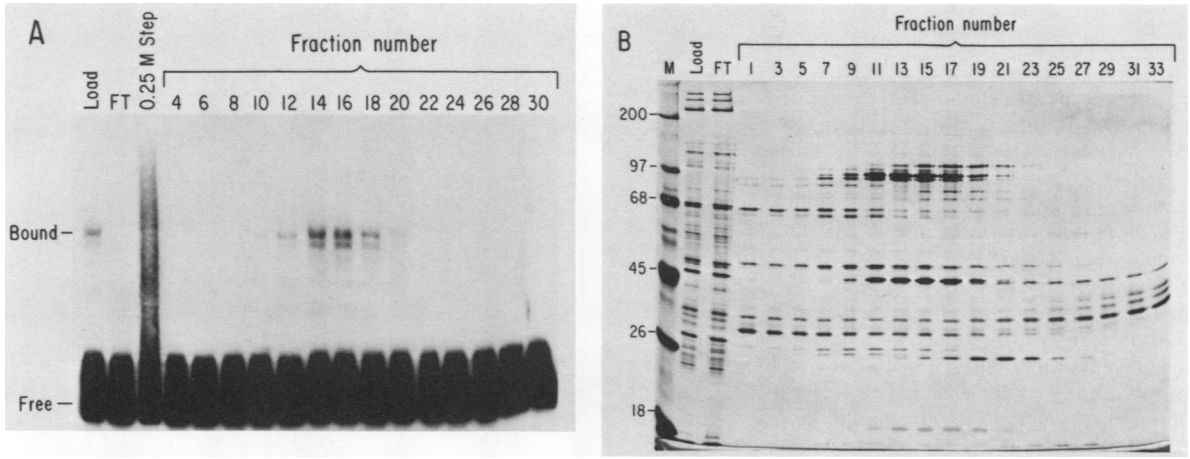

FIG. 2. Heparin-Sepharose gradient elution of MLTF activity. (A) DNA-binding assays. Binding reaction mixtures (10 μl) included 0.2 μg of poly(dI-dC) · (dI-dC), 2 μg of BSA, 0.3 ng of ML+, and either 3 μl of the load (DEAE-Sephacel flowthrough fraction AA), 3 μl of the heparin-Sepharose flowthrough (FT), 3 μl of the 0.25 M KCl wash, or 1 μl of the indicated heparin-Sepharose gradient fraction. (B) SDS-polyacrylamide gel analysis. Proteins were electrophoresed on a denaturing 12.5% polyacrylamide gel and silver stained. Lanes: M, Molecular weight standards; Load, 3 μl of DEAE-Sephacel flowthrough; FT, 3 μl of heparin-Sepharose flowthrough; 1 through 33, 5 μl of heparin-Sepharose fractions. Molecular weight standards are given adjacent to the gel.

ing the MLTF binding site (BML+) and a control fragment from a mutant MLP lacking the MLTF binding site (BML−). Peak fractions of MLTF activity from the heparin-Sepharose column were incubated with one of the two biotinylated DNA fragments, BML+ or BML−, under standard binding conditions. Control experiments demonstrated that the presence of the deoxynucleotide-biotin conjugate at one end of the probe did not affect the binding of MLTF (data not shown). When the MLTF binding reaction had reached equilibrium, streptavidin-agarose beads were added to each of the binding mixtures, and the incubation was continued until the binding of the biotinylated fragments to the streptavidin had gone to completion. The binding reaction was terminated by brief centrifugation to separate the streptavidin beads from the supernatant. Incubation with the biotinylated BML− control fragment left all the MLTF binding activity in the supernatant (Fig. 4B, lane 5). However, when the same procedure was performed with the biotinylated BML+ fragment, virtually 100% of MLTF binding activity was removed from the supernatant (lane 6). These results demonstrate that MLTF can be completely and specifically adsorbed onto a streptavidin-agarose matrix solely by virtue of its ability to bind to its target sequence.

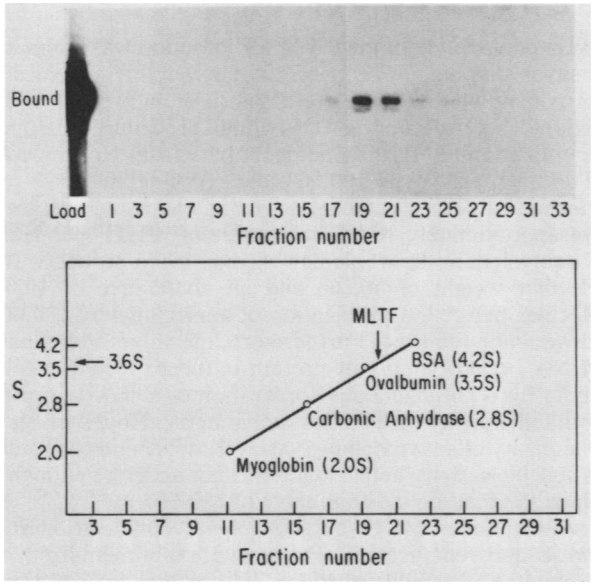

FIG. 3. Glycerol gradient sedimentation of MLTF. Heparin-Sepharose fractions containing MLTF were sedimented as described in Materials and Methods. Fractions (3 μl) were assayed for DNA-binding activity as described for Fig. 2. Lanes 1 and 33 correspond to the top and bottom of the gradient, respectively. Molecular weight standards were sedimented in parallel with the MLTF sample, equivalent fractions were collected, and the extent of sedimentation of the standards was determined by Coomassie blue staining of the SDS-polyacrylamide gel analysis of fractions. The lower panel shows the plot of S values of standards with respect to the peak fraction in which they sedimented.

Because of the extraordinarily high-affinity interaction between biotin and streptavidin, MLTF activity present on the BML+ beads could be eluted with increasingly stringent washes without releasing the DNA fragments bound to the column. The streptavidin-agarose beads were poured into a column and washed with elution buffers consisting of 0.1% Nonidet P-40 and either 0.15 or 0.5 M KCl. As expected, when the BML− streptavidin column was washed with either the 0.15 or 0.5 M KCl buffer, no MLTF activity was eluted (Fig. 4B, lanes 7 and 9). Similarly, when the BML+ streptavidin column was washed with the 0.15 M KCl buffer, no MLTF binding activity was eluted from the column (lane 8). However, when the BML+ column was eluted with the 0.5 M KCl buffer, MLTF binding activity was eluted (lane 10). Recovery of binding activity from the column was estimated to be between 30 and 40%. Thus, MLTF activity can be specifically adsorbed onto a streptavidin-agarose matrix and eluted in active form when the DNA fragment coupled to the matrix contains the MLTF binding site.

Proteins in each of the fractions recovered from the affinity chromatography steps were electrophoresed on SDS-polyacrylamide gels and visualized by silver staining. This analysis revealed that virtually all of the proteins in the heparin-Sepharose fraction remained in the original supernatant of the BML+ and BML− binding reaction mixtures

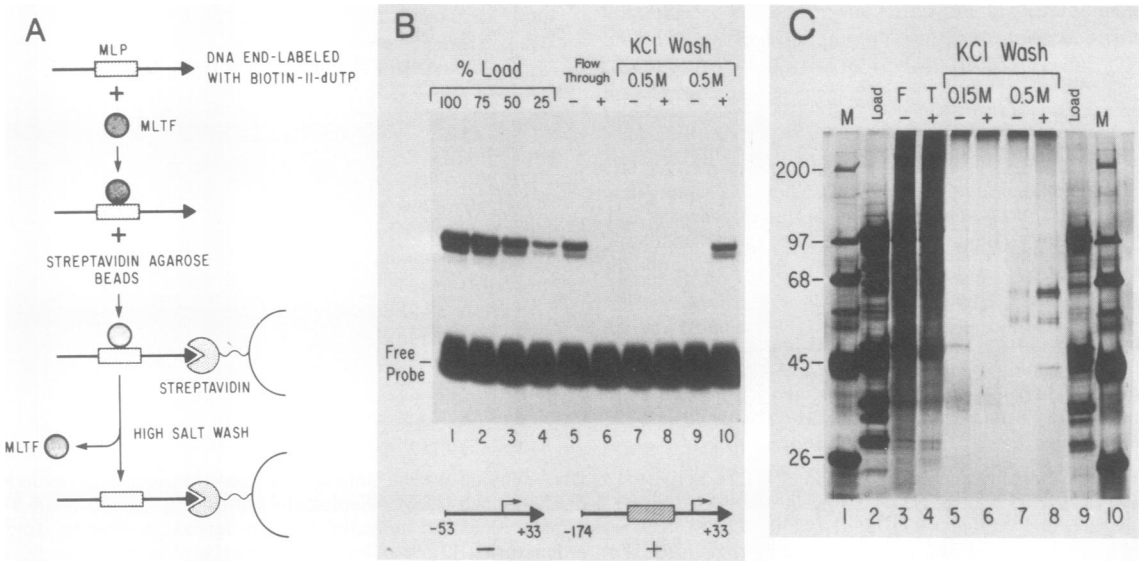

FIG. 4. DNA affinity chromatography of MLTF by biotin-streptavidin selection. (A) Scheme for DNA affinity purification. The DNA fragment containing the MLTF binding site was end labeled with biotin-11-dUTP. The resulting biotinylated fragment was incubated with MLTF-containing protein fractions and unlabeled carrier poly(dI-dC) · (dI-dC) to generate specific nucleoprotein complexes. The complexes were selected by binding to streptavidin-agarose. Contaminants were removed by washing the bound material, followed by elution of the purified MLTF with high-salt buffer. (B) DNA-binding assays. Binding reaction mixtures (10 μl) included 0.5 μg of poly(dI-dC) · (dI-dC), 1 μg of bovine insulin or BSA, 0.3 ng of ML+, and either an aliquot of the load (heparin-Sepharose fraction M3), 1.2 μl of the flowthrough from the DNA affinity matrix, or 1.2 μl of the indicated salt wash of the DNA affinity column. % Load refers to the expected DNA-binding activity if 100, 75, 50, or 25% of the input MLTF activity was recovered from the column. Chromatography was performed on a streptavidin-agarose matrix with either BML+ (lanes 6, 8, and 10) or BML− (lanes 5, 7, and 9) biotin-labeled DNA for the affinity selection. The biotin-labeled DNA fragments are shown below the gel. (C). SDS-polyacrylamide gel analysis. Proteins were electrophoresed on a denaturing 10% polyacrylamide gel and silver stained. Lanes: 1 (M), Molecular weight standards; 2 (Load), 2 or 4 μl of heparin-Sepharose fraction M3; 3 and 4 (FT), 15 μl of affinity column flowthrough fraction; 5 to 8, 750 μl of affinity column salt washes concentrated by precipitation in 10% trichloroacetic acid. Chromatography was performed with either BML+ (lanes 4, 6, and 8) or BML− (lanes 3, 5, and 7) biotin-labeled DNA for the affinity selection. Molecular weight standards are adjacent to the gel.

(Fig. 4C, lanes 2 to 4). Since the MLTF binding activity eluted only in the 0.5 M KCl wash of the BML+ beads, polypeptides with MLTF activity should be present in the 0.5 M KCl eluate of the BML+ column but absent from the corresponding 0.5 M KCl eluate of the BML− column. Only one major polypeptide was found in the 0.5 M KCl eluate of the BML+ column which was not found in the corresponding BML− fraction (lanes 7 and 8). This polypeptide had a molecular weight of 46,000. The faint band migrating with the mobility of a 98-kDa protein in the 0.5 M KCl eluate of the BML+ column was not observed in any other experiment, whereas the 46-kDa polypeptide was reproducibly observed in 12 independent experiments. Two sets of polypeptides in the 60- to 68-kDa range were present in both the 0.5 M KCl eluates of the BML+ and BML− columns. These bands were often observed with variable intensity, even in control reaction mixtures to which no cellular protein had been added. Most probably, these bands are generated by polypeptides of the keratin family, which commonly contaminate glassware and buffers. They are most likely present because these samples were trichloroacetic acid precipitated out of large volumes of buffer. Since the same bands were seen in fractions from both the BML+ and BML− columns, these bands are unlikely to be related to the MLTF binding activity. The streptavidin-biotin selection therefore confirms the identification of MLTF as a 46-kDa polypeptide, as previously suggested by photochemical cross-linking and glycerol gradient sedimentation analysis.

The streptavidin-biotin affinity column gave approximately a 250-fold purification when the concentration of the 46-kDa polypeptide in the 0.5 M KCl fraction was estimated by silver-staining intensity. This polypeptide constituted approximately 80% of the HeLa cell protein present in this fraction. Therefore, in the four-column procedure, the overall purification of MLTF binding activity from HeLa whole-cell extract was over 100,000-fold. A yield of 11% was obtained (Table 1). Previously, we had estimated that there were approximately 10,000 molecules of MLTF per HeLa cell equivalent in the whole-cell extract. For a protein with a molecular weight of 46,000 and an abundance of 10,000 molecules per cell, a purification of approximately 100,000-fold would be required. Furthermore, the value 22,600 pmol of DNA bound per mg of protein is the expected specific activity for a homogeneous preparation of a 46-kDa protein which binds DNA with a 1:1 stoichiometry. However, since the intensity of silver staining varies from protein to protein, this specific activity determination is not accurate enough to deduce the binding stoichiometry of MLTF.

Identification of MLTF by SDS-polyacrylamide gel electrophoresis and renaturation. The above experiments did not rule out the possibility that MLTF contains an additional polypeptide of low molecular weight which was required for binding activity. To exclude this possibility, a sample of affinity-purified MLTF (100,000-fold) was applied to an SDS-polyacrylamide gel. After electrophoresis, the gel was divided into 6-mm slices, and the protein in each slice was eluted and precipitated by acetone to remove the SDS, dissolved in 6 M guanidine hydrochloride, and diluted to permit renaturation (9). When proteins were eluted and renatured from gel slices corresponding to a wide range of molecular weights, only the slice which contained the poly-

peptide of molecular weight 46,000 showed MLTF binding activity (Fig. 5, lane 6). This demonstrates that the 46-kDa polypeptide alone possesses MLTF binding activity.

MLTF binding activity was also detected in gel slices corresponding to 46 kDa when unfractionated HeLa whole-cell extracts were resolved on SDS-polyacrylamide gels. This method provides a rapid and simple means of estimating the molecular weight of a sequence-specific DNA-binding protein, which can subsequently be corroborated by photochemical cross-linking, sedimentation analysis, and streptavidin-biotin selection.

Stimulation of transcription by purified MLTF. It was previously demonstrated that the intact adenovirus type 2 MLP is transcribed in HeLa whole-cell extracts approximately 20 times more efficiently than a MLP which lacks the MLTF binding site (4, 10, 13). When the mutant and wild-type promoters were assayed in a system reconstituted from a mixture of chromatographic fractions which lacked MLTF binding activity, there was no difference in the transcriptional activity of the two promoters (4). These results can also be seen in Fig. 6 (lanes 1 and 2). Addition to the reconstituted system of a small amount of unfractionated extract (lane 3) or any of the four column chromatographic fractions containing MLTF binding activity (lanes 4 to 7) resulted in the preferential transcription of the wild-type template. Therefore, MLTF binding and transcription-stimulatory activities copurified 100,000-fold through all four chromatographic steps.

The degree of transcription stimulation observed was in all cases proportional to the amount of MLTF binding activity added from each chromatographic fraction. Addition of whole-cell extract or fraction M3 to the reconstituted system resulted in a 15- to 20-fold preferential increase in transcription from the wild-type MLP (Fig. 6, lanes 3 and 6). Addition of 100,000-fold-purified MLTF binding activity to the reconstituted system yielded a fivefold preferential stimulation of transcription of the wild-type template (lane 7). The greater degree of stimulation observed in whole-cell extract and in fraction M3 relative to that in fractions A, AA, and M4 occurs because the MLTF binding activity is 3 to 100 times more concentrated in whole-cell extract and fraction M3 than in fractions A, AA, and M4. The apparent variability in the intensity and position of the Δ51 runoff transcript occurs because some of the lanes were taken from different autoradiographic exposures of different gels. Under no circumstances was Δ51 transcription stimulated by addition of MLTF.

Although MLTF transcription activity copurified with the 46-kDa polypeptide responsible for MLTF binding activity, we could not rule out the possibility that transcription stimulation requires an additional polypeptide. To address this possibility, we electrophoresed the proteins from the 100,000-fold affinity-purified MLTF fraction on an SDS-polyacrylamide gel and then eluted and renatured proteins from various regions of the gel. Proteins eluted from the gel in the molecular weight range of 44,000 to 48,000 preferentially stimulated transcription from the wild-type MLP (Fig. 6, lane 8). The degree of stimulation observed was the same as that expected on the basis of the amount of MLTF binding

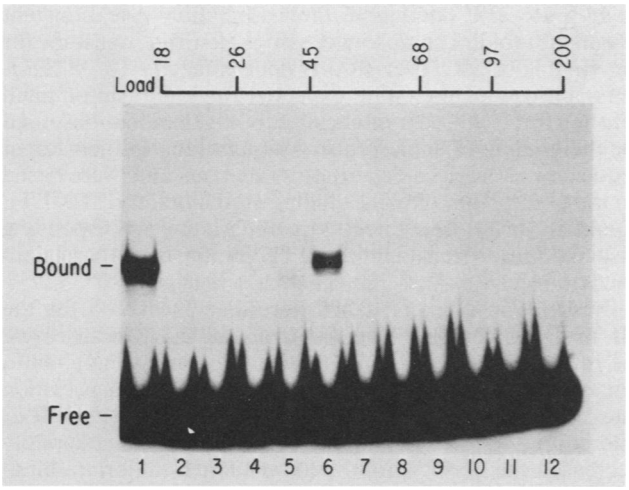

FIG. 5. Renaturation of gel-purified MLTF. DNA affinity chromatographic fractions containing MLTF (60 μl) were electrophoresed on a denaturing polyacrylamide gel, and proteins from individual gel slices were eluted and renatured as described in Materials and Methods. Eluate from each gel slice (5 μl) was assayed for DNA-binding activity. Binding reaction mixtures (20 μl) included 4.7 μg of BSA, 1 μg of poly(dI-dC) · (dI-dC), and 2 ng of ML+ DNA. The binding reaction mixture of the load (lane 1) included 0.06 μl of heparin-Sepharose fraction M3. Molecular weights denoted above the gel refer to the location of molecular weight protein standards relative to the gel slice boundaries. Protein standards were electrophoresed in lanes adjacent to the MLTF sample.

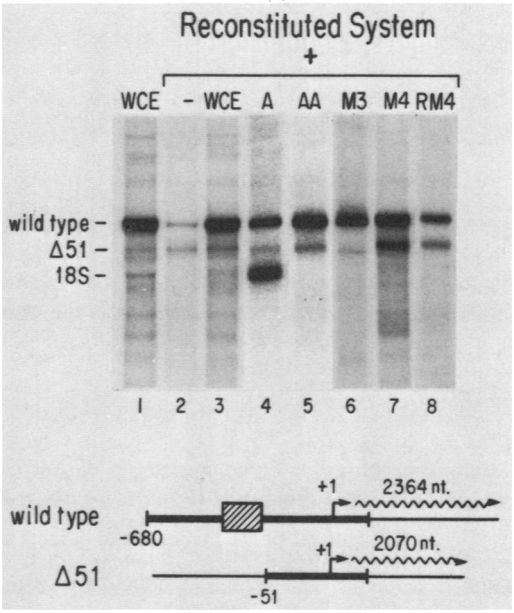

FIG. 6. Transcription analysis of MLTF fractions. Analytical transcriptions were performed as described in Materials and Methods. The DNA templates used are shown at the bottom of the figure and were described in detail previously (4). An arrow at 18S indicates the position of radioactively labeled rRNA. All reaction mixtures received an equimolar mixture of DNA templates and either 45% (vol/vol) HeLa whole-cell extract (72 U of MLTF DNA-binding activity) (lane 1) or 8 μl of a mixture of HeLa chromatographic fractions plus purified RNA polymerase II (lanes 2 to 8). Reconstituted system reaction mixtures were supplemented with no further addition (lane 2) or with 2 μl of whole-cell extract (16 U of MLTF DNA-binding activity) (lane 3), 5 μl of phosphocellulose fraction A (4.4 U of binding activity) (lane 4), 5 μl of DEAE-Sephacel fraction AA (24 U of binding activity) (lane 5), 2 μl of heparin-Sepharose fraction M3 (100 U of binding activity) (lane 6), 1.6 μl of DNA affinity column fraction M4 (8 U of binding activity) (lane 7), or 2 μl of gel-purified MLTF prepared as described for Fig. 5 (4 U of binding activity) (lane 8). One unit of binding activity is equivalent to 1 fmol of DNA fragment bound.

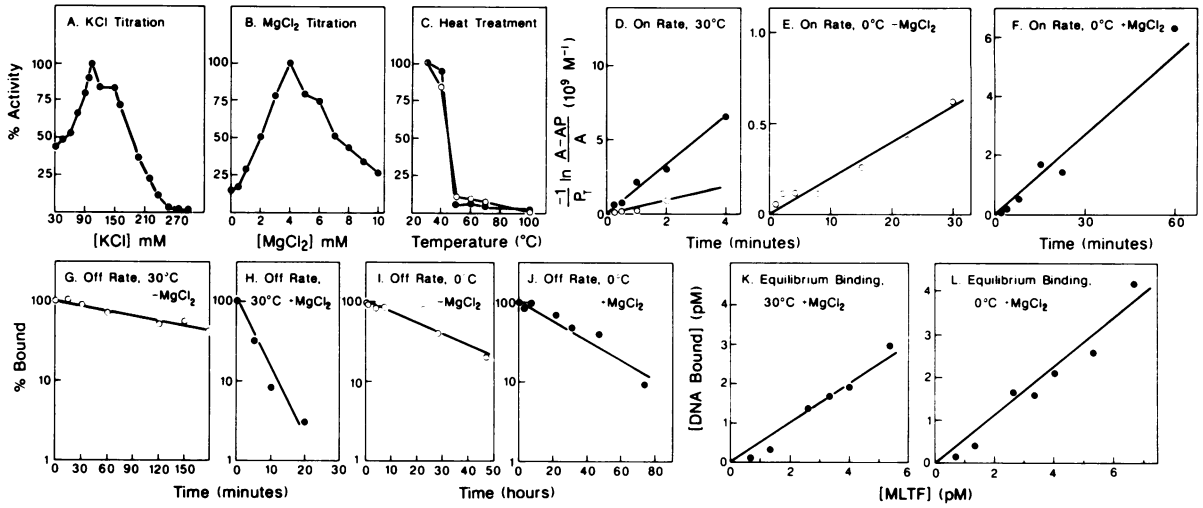

FIG. 7. Physical parameters of MLTF binding to DNA. The MLTF binding parameter measured is described inside each panel. Analytical binding reactions were performed in a 10-μl volume with 0.3 ng of ML+ fragment in a final concentration of 0.25 mg of BSA per ml under standard buffer conditions. The amount of DNA affinity column fraction M4 added to each reaction is indicated in Materials and Methods. Reactions were performed in the absence (○) or presence (●) of 5 mM $MgCl_2$. Reaction parameters, electrophoresis, and quantitation of binding products were as described in Materials and Methods.

activity added to the reaction. The only protein visible in this region of the gel was the 46-kDa polypeptide responsible for MLTF binding activity (Fig. 4C, lane 8). Preferential stimulation of transcription was not observed for material eluted from any other region of the gel (data not shown). Therefore, we conclude that the purified 46-kDa polypeptide is solely responsible for the promoter-specific binding and the transcription-stimulatory activities of MLTF.

Characterization of MLTF. The kinetic and thermodynamic properties of specific DNA binding by the most highly purified MLTF fraction were characterized. The fraction of probe DNA bound was determined by the gel electrophoresis DNA-binding assay. Since only purified MLTF was added to the reaction, addition of excess carrier DNA to quench the nonspecific binding of contaminating proteins was not necessary. Therefore, binding reaction mixtures did not contain any DNA other than probe DNA.

Figure 7A shows the effect of different KCl concentrations on the binding of MLTF to its target sequence. The apparent equilibrium binding constant increased twofold as the KCl concentration was raised from 30 to 105 mM. At KCl concentrations greater than 150 mM, the apparent binding constant K_{app} decreased dramatically. The slope of the plot of ln K_{app} versus ln [KCl] for binding reactions in the absence of $MgCl_2$ was linear in the range 150 to 300 mM KCl and had a slope of 9 ± 1, suggesting that approximately 10 monovalent ions are displaced upon binding of MLTF to its target sequence (25).

Titration of $MgCl_2$ concentration showed a K_{app} peak at 4 mM (Fig. 7B). The binding of MLTF decreased rapidly at concentrations higher than 4 mM $MgCl_2$.

Heat treatment of purified MLTF showed that binding activity was almost completely abolished after 5 min at temperatures above 40°C (Fig. 7C). This heat sensitivity was unaffected by the presence of $MgCl_2$ (Fig. 7C) or by the presence of DNA either containing or lacking the MLTF binding site (data not shown). These results contrast sharply with those of Sawadogo and Roeder (32), who found that MLTF activity survived incubation at 100°C for 5 min. It is possible that the heat stability which they observed was due to other components in their MLTF-containing fractions or to their use of conditions which allowed renaturation of the polypeptide.

The second-order rate constant for MLTF binding was estimated from the measured rate of MLTF binding by applying the equations of Riggs et al. (29) (Figures 7D to F; Table 2). In all cases, binding of MLTF to its target sequence was linear with respect to time, as expected for a second-order reaction in the presence of an excess of one reactant. The presence of 5 mM $MgCl_2$ increased the association rate constant three- to fivefold, while an increase in temperature from 0 to 30°C increased the association rate constant roughly 20-fold. The absolute association rate constant for the binding of MLTF at 30°C is approximately 10^7 $M^{-1}s^{-1}$. This approaches the value expected for a diffusion-limited reaction but is an order of magnitude less than that observed for the binding of some procaryotic proteins to their target sequences (27). All calculations of rate constants were based on the assumption of a 1:1 binding stoichiometry. If MLTF bound its target sequence as a dimer, the corresponding association rate constant would be greater than that calculated above.

Dissociation rate constants were also measured for the MLTF-DNA complex (Fig. 7G to J; Table 2). In all cases, the reactions appeared to be first order. Interestingly, addition of 5 mM $MgCl_2$ dramatically increased the dissociation rate constant at 30°C but had little or no effect at 0°C. Decreasing the temperature from 30 to 0°C moderately decreased the dissociation rate constant. Equilibrium bind-

TABLE 2. Kinetic and thermodynamic analysis of MLTF binding

Temp (°C)	$MgCl_2$	K_{eq} (M^{-1})	k_a (M^{-1} s^{-1})	k_d (s^{-1})	k_a/k_d (M^{-1})
0	−	ND	3.4×10^5	9.4×10^{-6}	3.6×10^{10}
	+	1.3×10^{10}	1.5×10^6	8.5×10^{-6}	1.8×10^{11}
30	−	ND	8.2×10^6	8.0×10^{-5}	1.0×10^{11}
	+	1.0×10^{10}	2.7×10^7	2.9×10^{-3}	9.2×10^9

[a] ND, Not determined.

ing constants calculated from the ratio of the kinetic rate constants were within the range of 10^{10} to 10^{11} M^{-1}.

Equilibrium binding constants were estimated by measuring the amount of MLTF-DNA complex formed as a function of MLTF concentration. In all binding reactions, the concentration of probe was much greater than that of MLTF (Fig. 7K and L; Table 2). Assuming a 1:1 binding stoichiometry, equilibrium binding constants in the presence of Mg^{2+} at 0 or 30°C were both approximately 10^{10} M^{-1} (Table 2). The equilibrium binding constant at 30°C corresponds to a binding free energy of −13.9 kcal/mol. Applying the van't Hoff equation $\ln (K_2/K_1) = \Delta H/R(1/T_2 - 1/T_1)$ resulted in the values $\Delta H = -1.1$ kcal/mol and $\Delta S = +42$ cal/mol per °C. Therefore, the binding of MLTF is primarily an entropically driven reaction.

Binding of purified MLTF to the synthetic alternating copolymers poly(dG-dC) · (dG-dC), poly(dI-dC) · (dI-dC), and poly(dA-dT) · (dA-dT) was examined by determining the amount of copolymer which would reduce by 50% the binding of MLTF to the MLP binding site. MLTF recognized the copolymers poly(dG-dC) · (dG-dC), poly(dI-dC) · (dI-dC), and poly(dA-dT) · (dA-dT) with 7.6×10^5-, 9.5×10^5-, and 2.8×10^6-times-lower affinity than the MLP binding site, respectively. The stronger binding of MLTF to poly(dG-dC) · (dG-dC) relative to poly(dA-dT) · (dA-dT) is possibly a reflection of the greater resemblance of the former to the binding site in the MLP.

DISCUSSION

We have described a rapid, high-yield purification scheme for MLTF, a transcription factor which specifically recognizes an upstream promoter element in the adenovirus MLP.

DNA affinity chromatography and three conventional chromatographic steps were used to purify MLTF DNA-binding activity 100,000-fold with a 11% recovery from HeLa whole-cell extract. Moreover, an activity that stimulated transcription in vitro from a template containing the MLTF binding site copurified with the sequence-specific DNA-binding activity. Thus the purified protein has all the biochemical properties previously attributed to MLTF.

An important aspect of this purification scheme was the unambiguous identification of the molecular weight of the polypeptide bearing MLTF activity. Results from four independent methods ascribe the binding activity of MLTF to a polypeptide of molecular weight 46,000 which was greatly enriched in the most highly purified fraction. First, UV cross-linking experiments with partially purified MLTF samples demonstrated that a 46-kDa protein was specifically cross-linked to radioactive probe DNA containing the MLTF binding site. Second, glycerol gradient sedimentation analysis established a native molecular weight for MLTF binding activity of 45,000 to 50,000. Third, a 46-kDa protein was specifically retained on a DNA affinity matrix only when the DNA fragment coupled to the matrix contained the MLTF binding site. Finally, proteins in the molecular weight range of 44,000 to 48,000 which were eluted and renatured from an SDS-polyacrylamide gel exhibited both MLTF binding and transcription-stimulatory activities. We thus conclude that this 46-kDa polypeptide is solely responsible for MLTF activity.

Several aspects of the biotin-streptavidin procedure make it useful as an affinity purification technique. In this approach, the factor binds to the specific target site in solution, thereby allowing reaction parameters to be easily optimized by the gel electrophoresis assay. Furthermore, the efficiency of binding per DNA molecule is high, since the column matrix cannot block the access of the protein to the DNA fragment. In addition, the binding capacity of streptavidin-agarose is high enough to potentially purify 20 to 30 nmol of DNA-binding protein per ml of column resin without necessitating the construction of DNA fragments containing tandemly repeated binding sites. Use of such constructs would further increase the capacity of the streptavidin-agarose matrix. Finally, the biotin-streptavidin DNA affinity technique can be easily manipulated for use on an analytical or preparative scale.

As an analytical technique, biotin-streptavidin DNA affinity selection may permit the direct identification of a wide variety of sequence-specific DNA-binding proteins. It has already been successfully used as an analytical method for identifying hormone receptors (8) and components in mRNA-splicing complexes (7a). If, as anticipated, the potential purification by DNA affinity selection is on the order of 1,000- to 10,000-fold, it should be possible to detect nonabundant DNA-binding proteins by affinity selection directly from soluble cell extracts followed by two-dimensional gel electrophoresis. Moreover, these proteins may be detected from extracts prepared from metabolically labeled cells.

The MLTF binding site (GTAGGCCACGTGACCGG in the MLP of adenovirus type 2) is highly conserved among the human adenoviruses (33, 35). The canonical binding site is characterized by a 12-bp imperfect palindromic sequence. Mutagenesis studies, as well as DNase I footprinting and methylation protection experiments, suggest that sequences in this palindrome play an important role in MLTF recognition (4, 18, 32, 36). Many procaryotic regulatory proteins specifically recognize palindromic DNA sequences as dimers (20, 24, 26). Although MLTF exist in solution as a monomer, it may be in the form of a dimer when bound to its palindromic recognition sequence. If this is the case, then the cooperativity between the two MLTF monomers upon binding must be quite high. This is suggested by the absence of a detectable DNA-MLTF monomer complex which would migrate more rapidly than the hypothetical dimer-DNA complex in the gel electrophoresis assay. A monomer-DNA complex with an equilibrium constant of greater than 10^7 M^{-1} would probably be detected in this assay. Alternatively, it is possible that only a monomer of MLTF binds to the apparently palindromic binding site. In this case, it is likely that further analysis of the sequence specificity of MLTF binding will reveal asymmetric interactions in the halves of the binding site.

The functioning of MLTF in vivo is probably based upon its physical occupancy of the MLTF binding site. The extent to which regulatory proteins can bind to their target sequences depends upon the affinities of these proteins for other cell components, including nontarget DNA (34). Estimates of the equilibrium constant for MLTF monomer binding to its target site fall between 1.0×10^{10} and 2.5×10^{11} M^{-1}. The affinity of MLTF for nonspecific binding sites was determined to be 6 orders of magnitude weaker than its affinity for specific binding sites. If the affinity of MLTF for specific versus nonspecific sites is the same in vivo as in vitro, the expression derived by Lin and Riggs (17) can be used to calculate the fractional occupancy of target DNA by MLTF. The concentration of MLTF in HeLa cells was estimated to be about 10,000 molecules per cell (4). This is equivalent to a concentration of 2.5×10^{-7} M, assuming that MLTF is confined to the nucleus. Given these parameters, an MLTF binding site in the promoter of a single-copy gene

would be occupied by MLTF 81% of the time. This calculation represents a minimal estimate of specific site occupancy, since it does not consider the masking of competing nonspecific sites by nucleosomes or the possibility of increased recognition specificity owing to cooperative interactions with other DNA-binding proteins. If there are 1,000 different genes per genome which contain an MLTF binding site, the degree of occupancy of each site would be only slightly reduced, to a value of 80%. Thus MLTF occupancy in the mammalian genome is determined primarily by the concentration of nonspecific binding sites and only secondarily by the concentration of specific binding sites. As a consequence, late in adenovirus infection, when the adenovirus genome copy number ranges from 1000 to 10,000, each MLP could theoretically be occupied approximately 80% of the time. Thus the activity of the MLP during lytic infection is not likely to be limited by MLTF availability.

The mechanism by which MLTF activates transcription from the MLP is unclear. Evidence suggests that MLTF comes in direct contact with the TATA box binding transcription factor (32). However, MLTF is able to bind with high affinity to the upstream element of the MLP in the absence of the TATA box binding factor (4, 32). Since one polypeptide possesses both MLTF transcription and DNA-binding activities, MLTF must have at least two functionally distinct domains, a DNA-binding domain and a domain which contacts another transcriptional factor (2, 12). Cloning of a cDNA copy of the mRNA encoding MLTF will allow genetic analysis of these two domains and will ultimately reveal the mechanism by which MLTF stimulates transcription.

ACKNOWLEDGMENTS

This work resulted from an equal contribution by the first two authors.

We thank A. Lamond, J. LeBowitz, and M. Gilman for critical reading of the manuscript and M. Siafaca for its expert preparation. We thank A. Fire and M. Samuels for preparation of extracts and some chromatographic fractions. We thank P. Grabowski for suggesting the use of biotin-streptavidin selection.

L.A.C. is supported by the Harvard Medical School M.D.-Ph.D. Program and by the Massachusetts Institute of Technology Department of Biology. This work was supported by Public Health Service grants CA38660 and PO1-CA42063 from the National Institutes of Health, grant CDR8500003 from the National Science Foundation, and, partially, NCI Cancer Center core grant P30-CA14051 to P.A.S.

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