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Isolation of a Novel cDNA Encoding a Zinc-Finger Protein That Binds to Two Sites within the c-*myc* Promoter[†,‡]

Janina J. Pyrc, Kenneth H. Moberg, and David J. Hall*
Department of Biochemistry and Molecular Biology, 208 LSB, Thomas Jefferson University, 233 South 10th Street, Philadelphia, Pennsylvania 19107

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ABSTRACT: The ME1a1 and ME1a2 elements are cis-acting DNA sequences that exist at positions −46 and −85, respectively, within the P2 promoter of the c-*myc* gene. These elements are required for optimal transcription initiation from P2. The proteins that bind to these elements are identical or very similar. Here we have isolated a cDNA clone that encodes the carboxy-terminal 494 amino acids of the human ME1a1/ME1a2 factor. This factor is referred to as "ZF87" (zinc-finger protein, 87 kilodaltons). ZF87 specifically binds the ME1a1 element with higher affinity than the ME1a2 element. Western blot analysis indicates that the full-length protein has a relative molecular mass of approximately 87 000 daltons, and Northern blot analysis shows that it is encoded by a 5-kb transcript. ZF87 contains six zinc-finger domains, of the Cys_2-His_2 type, at the carboxy terminus of the protein. The protein also contains extended tracts of polyalanine.

Expression of the c-*myc* gene plays an important role in controlling cell proliferation (Cole, 1986). One mechanism that is involved in regulating c-*myc* expression occurs at the level of transcription initiation. However, to fully understand how transcription initiation is regulated, it is necessary to characterize the elements that constitute the c-*myc* promoter.

A majority of transcripts initate from the P2 promoter of the c-*myc* gene (Cole, 1986). While the region distal to the P2 promoter contains a number of enhancer and repressor elements (Avigan et al., 1990; Hay et al., 1987; Iguchi-Ariga et al.; 1988; Kakkis et al., 1989; Weisinger et al., 1988), the proximal region of the P2 promoter is composed of three

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[‡] The amino acid sequence reported in this paper has been submitted to GenBank under Accession Number J05371.
* To whom correspondence should be addressed.

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elements in addition to the TATA box (Asselin et al., 1989; Hall, 1990). These are termed ME1a2, E2F and ME1a1 and are found at positions −85, −64, and −46, respectively. The E2F element was originally identified in the adenovirus E2 promoter (Kovesdi et al., 1986) and appears to be required for c-*myc* promoter activity in *Xenopus* oocytes (Nishikura, 1986) and for transactivation of the c-*myc* gene by the adenovirus E1a protein (Heibert et al., 1989; Lipp et al., 1989; Thalmeier et al., 1989). Recently, it has been shown that binding of E2F to its cognate sequence is regulated by cell growth conditions and that this regulated binding determines the extent of c-*myc* expression (Mudryj et al., 1990). The ME1a2 and ME1a1 elements have yet to be identified as functional elements in the promoters of other genes. It is known that deletion of each individual element reduces the extent of transcription initiation from P2 in vivo (Moberg et al., 1991, 1992). Further, the ME1a1 element has a small positive effect on increasing transcription initiation in an in vitro assay (Hall, 1990) and is necessary for initiation at P2 in vivo (Asselin et al., 1989). Additionally, evidence suggests that the ME1a1 element is essential for premature termination of transcription at the 3′ end of the first exon (Miller et al., 1989). Thus, these elements appear to have dual functions, by acting to regulate both initiation from P2 and premature termination at the end of the first exon.

To better understand the mechanisms by which these factors bind to the c-*myc* promoter and interact, it is essential that their cDNAs be isolated. Only then can the corresponding activation, interaction, and binding domains of these factors be characterized. Here we describe the isolation of a cDNA clone that encodes a protein that binds to the ME1a1 and ME1a2 sites within the c-*myc* promoter. The derived amino acid sequence of this factor predicts a zinc-finger-containing protein.

Materials and Methods

λgt11 Library Screening. A λgt11 cDNA library constructed from HeLa cell mRNA (a kind gift of Dr. Paula Henthorn, University of Pennsylvania) was used to screen for the ME1a2 binding factor. A total of 5×10^4 PFU were plated with *Escherichia coli* strain Y1090 on 150-mm plates and grown at 42 °C for 4–6 h until confluent plaques were obtained. The plates were overlayed with nitrocellulose filters, saturated with IPTG (10 mM), for 5 h at 37 °C. The filters were air-dried and processed for binding as previously described with modifications (Vinson et al., 1988). All subsequent steps were performed at 4 °C with gentle shaking. The filters were submerged for 5 min in binding buffer (20 mM Hepes, pH 7.9; 3 mM $MgCl_2$; 40 mM KCl; 1 mM DTT) containing 6 M guanidine hydrochloride, and the solution was removed and fresh binding buffer with 6 M guanidine hydrochloride added. This solution was diluted with an equal volume of binding buffer (without guanidine hydrochloride) for 5 min. The dilution steps were repeated four more times followed by two washes in binding buffer alone. The filters were then submerged in binding buffer alone with 5% Carnation nonfat dry milk for 30 min to block nonspecific sites and then hybridized with 1×10^6 cpm/mL ^{32}P-labeled probe (4 mL/filter) in 0.25% Carnation nonfat dry milk. The probe was generated by concatenating the double-stranded ME1a2 element (containing *Mbo*I ends) shown in Figure 1A. It was then ^{32}P-labeled by nick-translation.

Construction of Maltose Binding Protein Fusion, Generation of Antibodies, and in Vitro Transcription and Translation. The p-MAL-c plasmid (New England Biolabs), containing the gene for the maltose binding protein, malE, under control of the tac promoter, was used to generate a fusion protein with ZF87. The 3′ end (C-terminus) of the malE gene is linked in frame, through a polylinker, to the lacZα gene. The ZF87 cDNA containing flanking *Eco*RI sites was cloned into the *Eco*RI site (within the polylinker) of pMAL-c. The resulting fusion protein contains the maltose binding protein (MBP), linked to the amino terminus of ZF87 (now lacking lacZα).

Antibodies were generated to ZF87 by purification of MBP:ZF87 on an SDS–polyacrylamide gel. New Zealand White rabbits were immunized with polyacrylamide gel slices containing MBP:ZF87 followed by a boost 3 weeks later. Two weeks after the boost serum was collected.

In vitro transcription reactions were performed by cloning the ZF87 cDNA into the *Eco*RI site of a modified pGEM-2 vector. This vector has CACCATG linked immediately 5′ of and in frame with the *Eco*RI site. The pGEM-2:ZF87 was linearized with *Nhe*I. Sense ZF87 transcripts were generated by transcription with T7 RNA polymerase. Transcripts (315–325 ng) were used to generate the ZF87 protein in an in vitro translation reaction with a nuclease-treated rabbit reticulocyte lysate (35 μL, Promega) in a total reaction volume of 50 μL. [^{35}S]Methionine at 0.9 mCi/mL was also included in the reactions.

Generation of Extracts, Electrophoretic Mobility Shift Assays, and DNase I Footprinting Assays. To generate bacterial extracts containing the MBP:ZF87 protein, *E. coli* strain HB101 was transformed with MBP:ZF87. The cells were grown at 37 °C to 0.4 OD, and then the culture was treated with 0.3 mM IPTG for an additional 2 h at 37 °C. For Southwestern blot hybridizations the cells were pelleted and resuspended in SDS gel buffer (2.3% SDS, 0.7 M β-mercaptoethanol), boiled, and electrophoresed on an 8% polyacrylamide gel.

To generate bacterial extracts for the electrophoretic mobility shift assay (EMSA), the bacteria were grown as above in IPTG and resuspended in lysis buffer [10 mM Na_2HPO_4, pH 7; 30 mM NaCl; 0.25% Tween 20; 10 mM β-mercaptoethanol; 10 mM EDTA; 10 mM EGTA; pepstatin (2 μg/mL); leupeptin (1 μg/mL); and 0.4 mM PMSF]. The bacteria were subjected to a rapid freeze–thaw cycle and then sonicated for 1–3 min. Cell debris was removed by centrifugation at 14000*g* for 30 min and the supernatant saved.

Nuclear extracts were generated from HeLa cells by the method of Dignam et al. (1983) with the additional protease inhibitors: pepstatin (2 μg/mL) and leupeptin (1 μg/mL). EMSA's were performed essentially as described by Carthew et al. (1985). Briefly, 0.5 ng of a ^{32}P-end-labeled promoter fragment or double-stranded oligonucleotide was incubated with nuclear extracts from the various cell lines in the presence of 1–3 μg of sheared salmon sperm DNA as a nonspecific competitor. The final buffer conditions for protein binding to the radiolabeled DNA were as follows: 16 mM Hepes, pH 7.9, 16% glycerol, 80 mM KCl, 0.16 mM EDTA, 0.4 mM DTT, and 0.4 mM PMSF. The reactions were then electrophoresed in a low-ionic strength (7 mM Tris, pH 7.9, 3.3 mM sodium acetate, 1 mM EDTA) 4% polyacrylamide gel and visualized by autoradiography.

DNase I footprinting assays were performed as in Hall (1990). Briefly, 0.5 ng of end-labeled promoter fragment (−140 to −24) was incubated with 0.1–1 μL of MBP:ZF87 (2 μg/μL) purified by amylose resin chromatography (New England Biolabs) using EMSA binding conditions without nonspecific DNA. Binding took place at room temperature for 2 min, followed by addition of $MgCl_2$ and $CaCl_2$ (6 and 2 mM final concentrations, respectively). DNase I (Boehringer

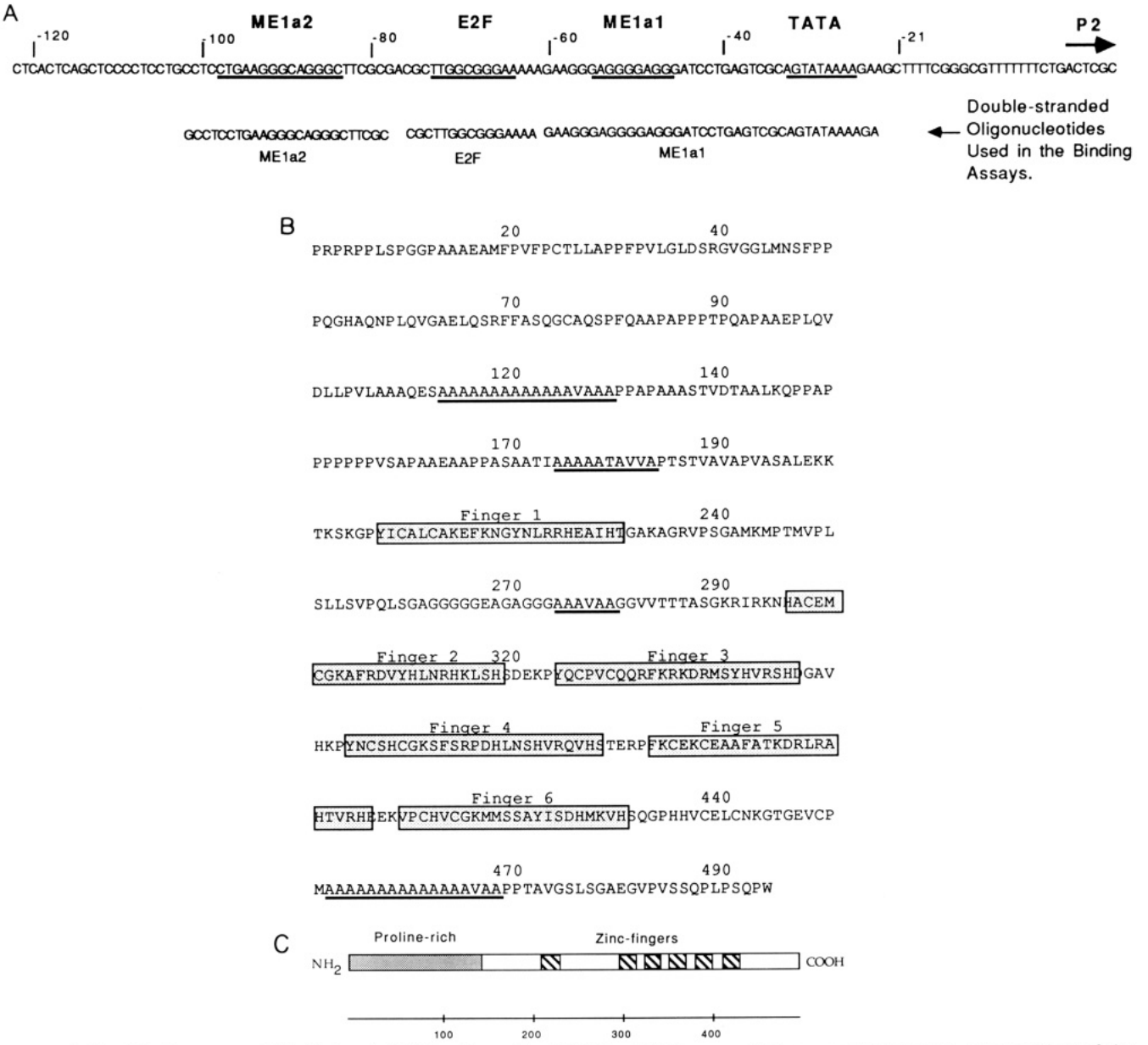

FIGURE 1: Predicted sequence of the C-terminal 494 amino acids of ZF87. (A) Diagram of the c-*myc* P2 promoter. The positions of the three promoter binding factors are underlined. The sequences of the double-stranded oligonucleotides used in the screen, the Southwestern blot hybridization, and the electrophoretic mobility shift assays (EMSA's) are shown. The ME1a2 element was concatermized, ^{32}P labeled, and then used as a probe to screen a λgt11 library. (B) Amino acid sequence of ZF87 isolated from a λgt11 library screened with radiolabeled ME1a2 element. The underlined regions denote the polyalanine tracts. The shaded boxed regions show the location of six zinc fingers. (C) Domains of the 494 NH_2-terminal amino acid residues of ZF87. The proline-rich regions of the protein are indicated by a shaded box. The zinc fingers are indicated by diagonal striped boxes.

Mannheim) was added to 3 units/mL final for 60 s. Reactions were stopped, phenol extracted, ethanol precipitated, and electrophoresed as described (Hall, 1990).

Northern, Western, and Southwestern Blot Hybridizations. For Northern blot hybridization, 3 μg of HeLa cell poly(A^+) mRNA and 20 μg of NIH3T3 fibroblast total RNA were electrophoresed on a 1% formaldehyde agarose gel and blotted onto nitrocellulose. The blot was hybridized by the method of Church and Gilbert (1984) with 1×10^6 cpm/mL ^{32}P-labeled ZF87 cDNA probe.

Western blot hybridizations were performed by electrophoresing 10 and 25 μg of HeLa nuclear extract on an 8% SDS–polyacrylamide gel. The proteins were blotted onto nitrocellulose which was then washed in TBST buffer (10 mM Tris, pH 8; 150 mM NaCl; 0.05% Tween 20). The blot was blocked with 1% BSA in TBST for 1.5 h at room temperature and then incubated with a 1:4000 dilution of primary antibody for 30 min at room temperature in TBST. The blot was incubated with a 1:7500 dilution of secondary antibody conjugated to alkaline phosphatase for 30 min at room temperature in TBST. The blot was then stained using the Protoblot system from Promega.

Southwestern blot hybridizations were performed by electrophoresing bacterial extracts on an 8% SDS–polyacrylamide gel and then blotting the proteins onto nitrocellulose. The blot was then subjected to guanidine hydrochloride denaturation and renaturation (i.e., by dilution) exactly as described for screening the λgt11 library. The appropriate ^{32}P-end-labeled probe (1×10^6 cpm/mL) was added to the filter overnight at 4 °C. Following binding the filters were washed in binding buffer plus 0.25% Carnation nonfat dry milk at 4 °C.

RESULTS

Isolation of a cDNA Clone for a c-myc Promoter Binding

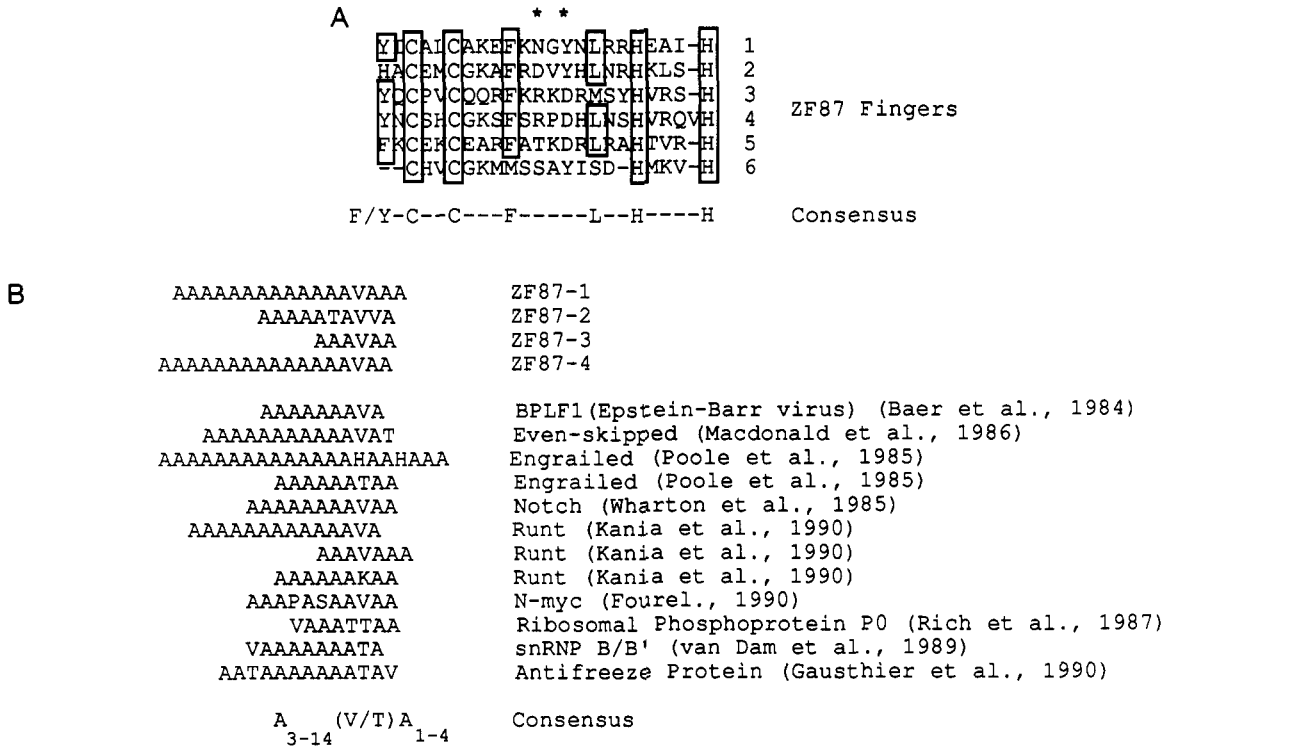

FIGURE 2: Alignments of the zinc fingers and the polyalanine tracts in ZF87. (A) Alignments of the six zinc fingers with the consensus. The exact positions of the zinc fingers are shown in Figure 1B. The asterisks denote the arginine and aspartic acid in fingers 3 and 4 that are identical to those in the zinc-finger protein Zif268 (Pavletich & Pabo, 1991). (B) Alignments of the polyalanine tracts in ZF87 to those in found in known proteins. The exact positions of the four alanine tracts are shown in Figure 1B (tracts 1–4 begin at amino acids 113, 174, 274, and 452, respectively).

Factor (ZF87) Reveals a Protein Containing Six Zinc Fingers and Alanine Repeats. A diagram of the c-*myc* P2 promoter with the locations of the major protein binding sites is shown in Figure 1A. The ME1a2 element is at the most 5′ position within the promoter. It has been shown that deletion of the ME1a2 element in c-*myc* promoter:CAT constructs significantly reduces transcription from P2 (Moberg et al., 1991, 1992). Also, a double-stranded oligonucleotide representing the ME1a2 element used in an electrophoretic mobility shift assay (EMSA) results in strong specific binding (Moberg et al., 1991). These data indicate that binding of the ME1a2 factor to its site is an important component of the P2 promoter. The isolation of the cDNA for the ME1a2 factor is therefore essential if one is to understand how transcription from P2 is regulated.

To isolate the cDNA for the ME1a2 factor, a HeLa cDNA library, cloned in λgt11, was screened with a ^{32}P-labeled, concatemerized form of the ME1a2 element. A single clone was isolated after screening approximately 1.5 million plaques. This clone remained positive through a tertiary screen and is referred to as "ZF87", for zinc-finger protein of 87 kilodaltons (see below). DNA sequencing showed that this clone is approximately 1800 bases in length and contains only one very long open reading frame of 494 amino acids, which is shown in Figure 1B. It is likely that this is only a partial cDNA due to the size of the full-length mRNA transcript and protein and the absence of an AUG or 5′ untranslated sequence (see Figures 3 and 5 below). The sequence predicts six consensus zinc fingers, of which five are clustered at the carboxy terminus. A proline-rich stretch of amino acids (24% in the first 167 amino acids) also exists at the amino acid terminus. The positions of the proline-rich stretch and zinc fingers are schematically represented in Figure 1C. Searches in both GenBank and the protein databases produced no exact homologies with other genes, with the exception of the zinc-finger domains, indicating that this is a unique protein.

The zinc fingers in ZF87 are of the Cys_2-His_2 type (Evans & Hollenberg, 1988; Miller et al., 1985) and are diagrammed in Figure 2A along with the consensus sequence. The asterisks denote an arginine and an aspartic acid in the third and fourth zinc finger that correspond exactly to those in the zinc-finger protein Zif268 that are essential for binding of Zif268 to DNA (Pavletich & Pabo, 1991).

An additional interesting feature of the predicted amino acid sequence is the presence of lengthy alanine repeats, shown in Figure 2B. These stretches of alanines are predicted to form α-helical conformations. A list of a known proteins containing polyalanine tracts is shown in Figure 2B along with a consensus.

Northern Blot Analysis Reveals That ZF87 Is Encoded by a 5-kb Transcript. The size of the ZF87 mRNA transcript was determined by Northern blot hybridization as shown in Figure 3. Three micrograms of HeLa poly(A$^+$) mRNA and 20 μg of total NIH3T3 fibroblast RNA were electrophoresed on a formaldehyde–agarose gel blotted onto nitrocellulose and probed with ^{32}P-labeled ZF87 cDNA. A strong hybridizing band was apparent only in the lane containing the HeLa poly(A$^+$) mRNA. This band migrates at a position coincident with the 28S ribosomal band from the adjacent lanes containing the NIH3T3 RNA. This indicates that the full-length transcript is roughly 5 kb in size.

A Fusion of ZF87 to a Maltose Binding Protein Produces a Protein That Binds the ME1a2 Element. To facilitate the study of the ZF87 protein, it was fused in both orientations to the carboxy terminus of a bacterial maltose binding protein (MBP). This construct is under transcriptional control of the tac promoter, rendering the gene inducible by IPTG. As shown in the SDS–polyacrylamide gel in Figure 4A, induction of an MBP:ZF87 fusion (ZF87 in the forward orientation) results in the production of a protein of over 100 kilodaltons

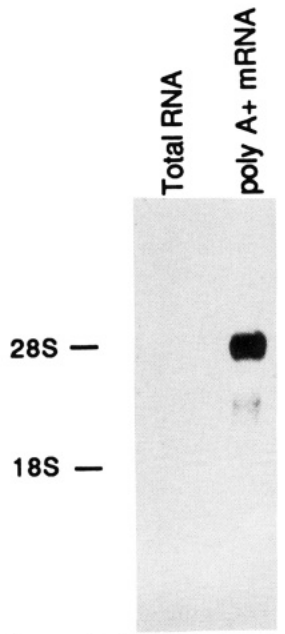

FIGURE 3: The ZF87 transcript is 5 kb in length. Northern blot of ZF87 mRNA. Three micrograms of poly(A^+) RNA from HeLa cells and 20 μg of total RNA from NIH3T3 fibroblasts were electrophoresed, blotted onto nitrocellulose, and probed with ^{32}P-labeled ZF87 cDNA. Shown are the positions of the 18S and 28S ribosomal bands from the lane containing the NIH3T3 total RNA.

(kDa). Induction of the MBP:ZF87 fusion with ZF87 in the reverse orientation results in a much smaller protein (42 000 daltons) due to a translation stop codon at the second codon in ZF87. The full-length MBP protein fused to Lac$Z\alpha$ is 55 kDa. These data indicate that the cDNA encoding ZF87 contains an open reading frame in the forward orientation which specifies a peptide of 55–60 kDa.

To test whether or not the MBP:ZF87 fusion binds to the ME1a2 element, an SDS–polyacrylamide gel identical to the one in Figure 4A was blotted onto nitrocellulose and the proteins were renatured with guanidine. The blot was then screened with a radiolabeled double-stranded ME1a2 element. An autoradiograph of this blot is shown in Figure 4B. It is clear that significant binding is achieved in the precise region of the blot corresponding to the MBP:ZF87 fusion protein, only with ZF87 in the forward orientation and only after induction by IPTG. No binding was observed to any bacterial protein or to MBP alone.

Western Blot Analysis Reveals That ZF87 Is an 87 000-Dalton Protein. To determine the size of the complete ZF87 protein, antibodies were generated to SDS–PAGE-purified MBP:ZF87. HeLa cell nuclear extracts were then electrophoresed by SDS–PAGE and then blotted onto nitrocellulose, as shown in Figure 5. Identical blots were incubated with either preimmune serum or MBP:ZF87-specific serum. As can be seen from the figure, the immune serum recognizes a protein of molecular mass 87 kDa that is not recognized by the preimmune serum (molecular mass estimation was made on the basis of Bio-Rad's unstained markers). The size of this protein is consistent with the size of the native ME1a2 factor derived from HeLa cells, as determined by UV-cross-linking experiments (a radiolabeled ME1a2 element covalently cross-linked to the HeLa ME1a2 protein; Moberg et al., 1992).

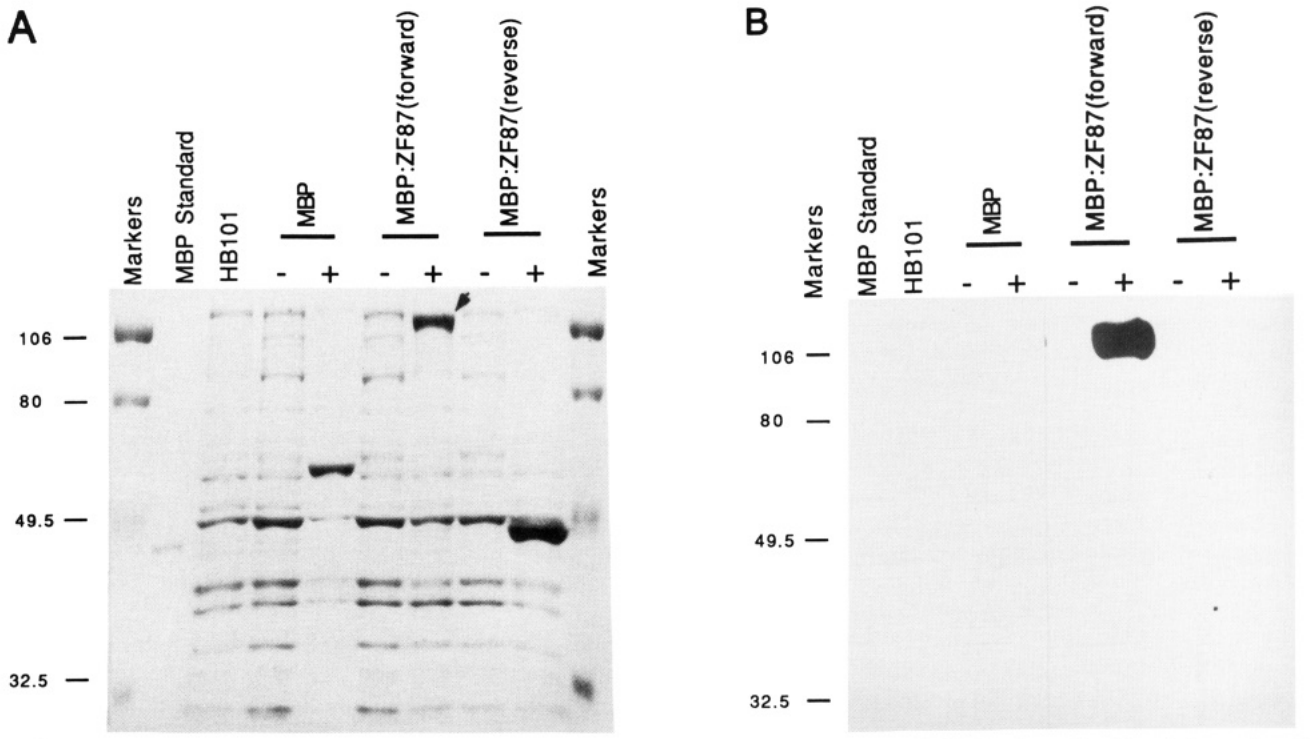

FIGURE 4: ZF87 fused to a bacterial maltose binding protein (MBP) binds to the ME1a2 element. (A) SDS–polyacrylamide gel electrophoresis of the bacterially produced MBP:ZF87 fusions. ZF87 was linked in the forward or reverse orientation between the maltose binding protein gene (MBP) and the Lac$Z\alpha$ gene. Following treatment with (+) or without (−) IPTG for 2 h, bacterial pellets were lysed directly in SDS buffer, boiled for 3 min, and electrophoresed on an 8% gel. The Coomassie blue-stained gel is shown. The molecular weight markers are Bio-Rad's Prestained Protein Standards. The arrow points to the full-length fusion protein. In the absence of any cloned gene, induction produces a fusion protein of MBP and Lac$Z\alpha$ (approximately 55 kDa). In the presence of a cloned gene (between MBP and Lac$Z\alpha$) a translation stop codon within the gene prevents translation of Lac$Z\alpha$. One lane contains extracts from bacteria only (HB101) while the lane marked "MBP" contains only the maltose binding protein alone (42 kDa). (B) Southwestern blot hybridization. A gel identical to the one in (A) above was electrophoresed, and the proteins were blotted onto nitrocellulose. The proteins on the blot were renatured by guanidine hydrochloride dilution. The blot was then probed with a ^{32}P-end-labeled ME1a2 element, overnight at 4 °C, and then washed and exposed to X-ray film. The autoradiograph is shown.

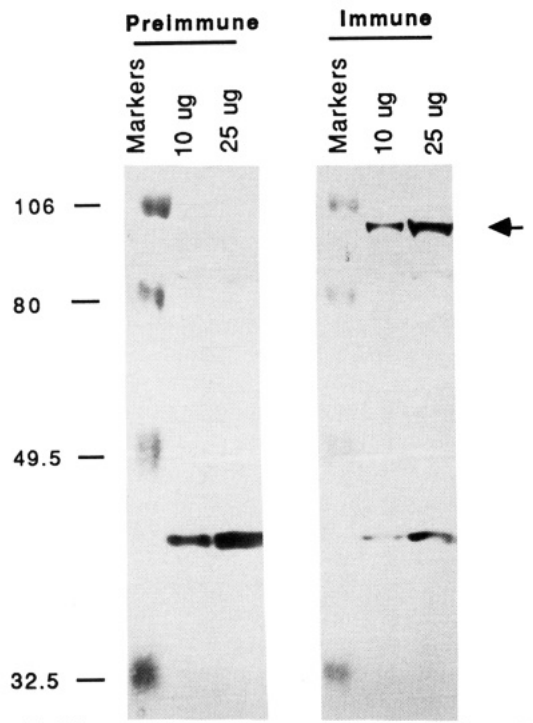

FIGURE 5: Western blot binding reveals that ZF87 is an 87-kDa protein. Ten- and twenty-five-microgram samples of HeLa cell nuclear proteins were electrophoresed on an 8% SDS–polyacrylamide gel. The proteins were immediately blotted onto nitrocellulose. Identical blots were incubated with either ZF87 antiserum (raised in rabbits against MBP:ZF87) or preimmune serum. Following incubation with a second antibody conjugated to alkaline phosphatase, a nitroblue tetrazolium staining reaction was performed. Shown are the stained blots. The arrow marks the position of the 87-kDa protein band specific for the immune serum. The molecular weight markers are Bio-Rad's Prestained Protein Standards.

Since the polyclonal antibody recognizes MBP in addition to ZF87, it is possible that the 87-kDa protein is a eukaryotic homologue of MBP. To determine if this was the case, a blot containing HeLa cell protein was incubated with antibody generated to MBP alone. The 87-kDa band was not recognized by the MBP-specific antibody, indicating that it is not a homologue of MBP (data not shown).

The results of the Northern and Western analyses indicate that the ZF87 cDNA is a partial length clone. The cDNA contains roughly two-thirds of the coding sequence and half of the transcript length. While we have recently isolated a number of additional cDNA clones for ZF87, none of them are full length. This may be due to the extraordinarily GC-rich region (approximately 90%) of the mRNA encoding the amino-terminal portion of the protein, which is known to be difficult for reverse transcriptase to completely transcribe.

ZF87 Binds Specifically and with High Affinity to the ME1a1 Element. To test the specificity of ZF87 binding to c-*myc* DNA, the MBP:ZF87 fusion was electrophoresed by SDS–PAGE, blotted onto nitrocellulose, renatured, and screened with a variety of ^{32}P double-stranded oligonucleotides. These oligonucleotides represent the binding site for the ME1a2, E2F, and ME1a1 factors (from the c-*myc* P2 promoter) as well as the *ter* site which represents the bacterial transcription termination sequence (CATAAAATAAGTATGTTGTAACTAAAGTG; Hill et al., 1988). It is clear from Figure 6 that the ME1a1 probe binds very strongly with the ZF87 fusion protein. In comparison, the ME1a2 element binds modestly; there is 35-fold more ME1a1 probe bound to the fusion protein than ME1a2 probe. The *ter* and E2F probes did not show any detectable binding. These data indicate that

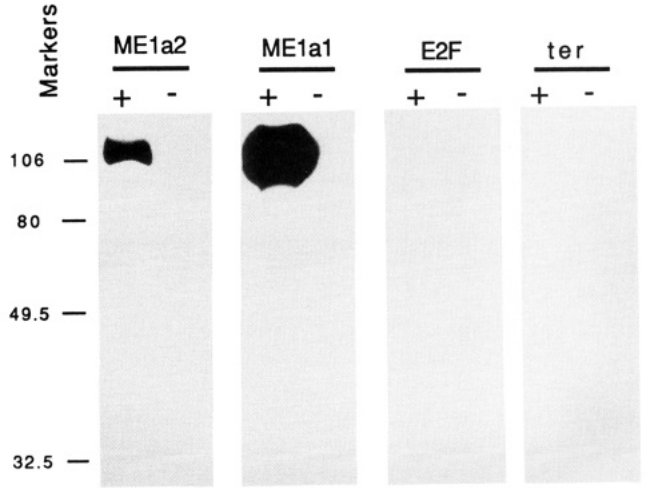

FIGURE 6: The ME1a1 element binds with higher affinity to MBP:ZF87 than the ME1a2 element. Southwestern blot binding. Bacteria containing the MBP:ZF87 construct (forward orientation) were treated with (+) or without (−) IPTG for 2 h, then lysed directly in an SDS sample buffer, and electrophoresed on an 8% polyacrylamide gel. The proteins were blotted onto nitrocellulose, renatured by guanidine hydrochloride dilution, and then screened to ^{32}P DNA represented by the ME1a2, ME1a1, E2F, or *ter* elements. Following the binding the blots were washed and exposed to film. The autoradiographs are shown. The molecular weight markers are Bio-Rad's Prestained Protein Standards.

while ZF87 recognizes both the ME1a2 and ME1a1 motifs, the latter binds with a greater affinity.

It should be noted that we have utilized a shorter ME1a1 element in the Southwestern hybridizations and EMSA's. This element is identical to the one in Figure 1A but does not contain the TATA sequence (i.e., does not contain GTATAAAAGA at the 3′ end). When used in the EMSA's and Southwestern hybridizations, the shorter element was found to bind to the HeLa cell factor, MBP:ZF87, and the in vitro translated ZF87 bound just as effectively as to the longer sequence (data not shown).

The ZF87 recognizes both the ME1a1 and ME1a2 sites is consistent with the recent finding that the ME1a1 and ME1a2 sites bind the identical or a very similar factor (Moberg et al., 1992). The consensus sequence for the ME1a1 and ME1a2 binding sites is

```
GAAGGGCAGGGCTTC ME1a2
I IIIIOIIII OII
GGAGGGGAGGG-ATC ME1a1
```

An alignment of the ME1a1 and ME1a2 binding sites, shown above, produces a match of 11 out of 15 bases over the entire binding site and 8 out of 9 in the center core of the elements (transversions are indicated by an oval) (Moberg et al., 1992).

To investigate the interaction of MBP:ZF87 with c-*myc* DNA in solution, extracts containing the MBP:ZF87 fusion protein were generated from bacteria following induction by IPTG. As shown in Figure 7 (left panel), the MBP:ZF87 band migrates faster than that produced by the wild-type protein. Since migration in the EMSA is dependent upon a number of factors (e.g., net charge, size, conformation), the difference in migration rates seen here is to be expected given that the characteristics of MBP:ZF87 are probably distinct from those of full-length ZF87.

To test for specificity of binding, the EMSA was performed with recombinant extracts (MBP:ZF87) and a radiolabeled ME1a1 probe with the addition of excess unlabeled competitor DNAs (Figure 7, right panel). Addition of excess unlabeled ME1a1 element significantly reduces binding while addition of excess unlabeled E2F element has no effect on binding.

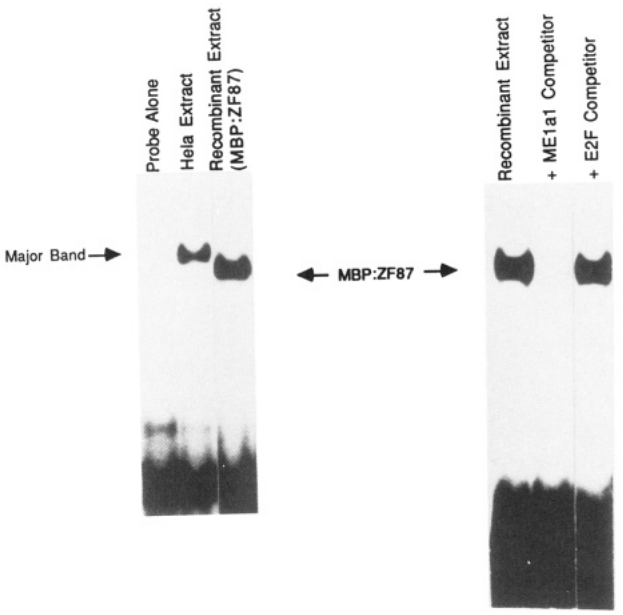

FIGURE 7: Recombinant MBP:ZF87 binds specifically to the ME1a1 element. (Left panel) Bacterial containing the MBP:ZF87 construct (forward orientation) were treated with IPTG for 2 h and then lysed by sonication. Bacterial debris was removed, and the soluble extracts were used in an EMSA with a ^{32}P-labeled ME1a1 element (as in Figure 7). Also included was a binding reaction using HeLa cell nuclear extracts. The position of the major band in the lane containing the HeLa extracts is indicated. The lane marked probe alone contained no extract. The intense band at the bottom is unbound probe. (Right panel) Bacterial extracts containing MBP:ZF87 were used in an EMSA with radiolabeled ME1a1 element as described above. Also included in the reactions were 100-fold excess unlabeled ME1a1 or E2F elements as competitors. The position of the shifted band corresponding to MBP:ZF87 is indicated.

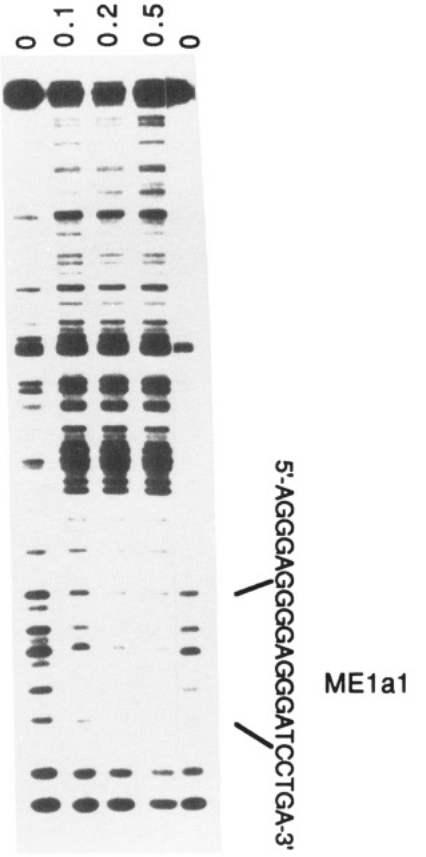

FIGURE 8: DNase I footprinting of purified MBP:ZF87 indicates binding to the ME1a1 element. An end-labeled promoter fragment (−140 to −24) was incubated with MBP:ZF87 and then used in a DNase I footprinting assay. The volumes of purified MBP:ZF87 (in microliters) from the amylose resin are indicated above the lanes. The region encompassing the ME1a1 element is indicated as is the footprint. Sequencing reactions electrophoresed next to the footprinting reactions were used to determine the precise position of the footprint within the labeled promoter element (not shown).

Bacterial extracts alone showed no binding activity (data not shown). These data indicate that binding of the ME1a1 element to the MBP:ZF87 is specific.

DNase I footprinting assays were performed to determine if MBP:ZF87 bound to the ME1a1 element on the promoter. As shown in Figure 8, MBP:ZF87, purified over an amylose resin, was used in the footprinting assay with an end-labeled promoter fragment (−140 to −24). The footprint appears over the ME1a1 element, indicating that this is the target sequence.

Under a variety of conditions we were unable to demonstrate that the ME1a2 element bound to recombinant MBP:ZF87 in the EMSA. It is possible that additional protein sequence is required for optimal binding to the ME1a2 site.

In Vitro Translated ZF87 Binds Specifically to the ME1a1 Element. To demonstrate that the 494 amino acid protein encoded by the ZF87 is capable of binding to the ME1a1 element independently of the maltose binding protein, the ZF87 protein was generated by in vitro translation and then used in the EMSA. The ZF87 cDNA was ligated into an in vitro expression vector in either the forward or reverse orientation in frame with an initiation codon in the pGEM-2 vector. RNA, generated by transcription with T7 RNA polymerase, was subsequently translated in a rabbit reticulocyte lysate with [^{35}S]methionine. The translation products are shown in Figure 9A. Translation of RNA generated from transcription in the forward orientation produced a prominent band that migrates at approximately 56 kDa. Translation of RNA from the reverse orientation produced a faint band at about 47 kDa which is also contained in the lane with no added RNA.

Following translation, the extracts were used directly in the EMSA with a radiolabeled ME1a1 element, as shown in Figure 9B. The in vitro translated extracts obtained using the forward orientation ZF87 produce two bands. The upper band appears only when RNA is added to the reaction. This upper band migrates faster than the band produced by the MBP:ZF87 protein or the HeLa factor, which is consistent with the fact that the protein is not full length. When excess unlabeled competitor is added, the ZF87 band is eliminated. The lower band appears to be derived from the reticulocyte lysates. These data indicate that ZF87 alone, when not part of a fusion protein, is capable of specifically binding to the ME1a1 element. ZF87 transcribed and translated in the reverse orientation did not produce any unique bands in the EMSA (data not shown).

Discussion

Here we describe the isolation of a novel cDNA clone for a c-*myc* promoter binding protein, termed ZF87 (zinc-finger protein, 87 kilodaltons). This clone was isolated from a λgt11 expression cDNA library on the basis of the ability of the expressed factor to bind to the ME1a2 element present in the c-*myc* P2 promoter. The size of full-length ZF87, 87 kDa, is very similar to the estimated size of the HeLa cell ME1a2 factor which has been identified by UV-crosslinking experiments [although originally identified as 94 kDa by Moberg et al. (1992), the molecular mass of the ME1a2 factor has been reassessed at approximately 85 kDa; Moberg and Hall, unpublished observations].

During the course of the studies described here it was found

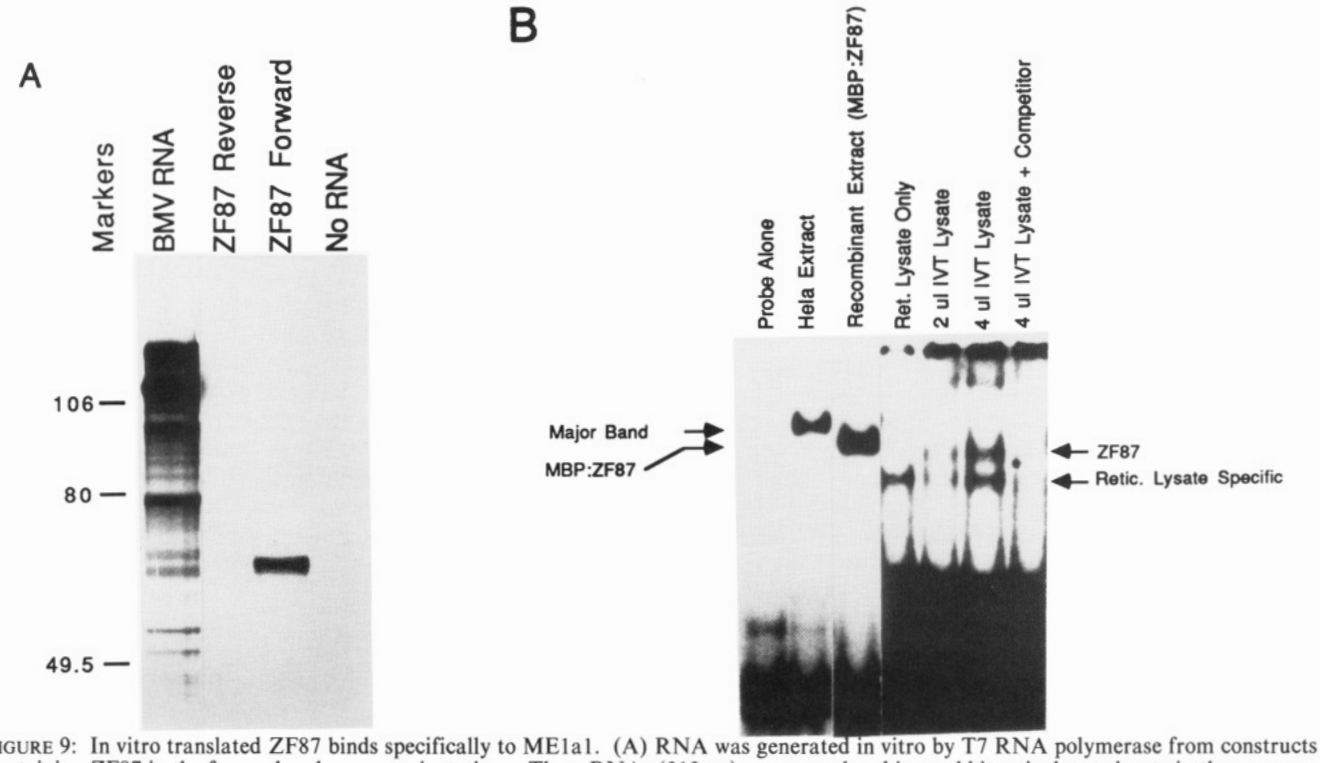

FIGURE 9: In vitro translated ZF87 binds specifically to ME1a1. (A) RNA was generated in vitro by T7 RNA polymerase from constructs containing ZF87 in the forward and reverse orientations. These RNAs (312 ng) were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. These extracts were electrophoresed on an 8% SDS–polyacrylamide gel, dried, and exposed to X-ray film. The autoradiograph is shown. Also included were reactions containing brome mosaic virus RNA ("BMV RNA") or water in place of RNA ("No RNA"). The molecular weight markers are Bio-Rad's Prestained Protein Standards. (B) An EMSA of in vitro translated RNA. A total of 325 ng of ZF87 in the forward orientation was translated in rabbit reticulocyte lysates. Two or four microliters of the in vitro translated lysate ("IVT Lysate") was used in an EMSA with a ^{32}P-labeled ME1a1 element. Included on the gel were binding reactions containing HeLa extract and recombinant MBP:ZF87 extract. All reactions contained 1 μg of salmon sperm DNA as a nonspecific competitor. The positions of the bands are indicated. Included in the reactions, where indicated, is 100-fold excess unlabeled ME1a1 competitor DNA. The reticulocyte lysate also contains a binding activity that is able to be competed by excess unlabeled ME1a1 element. Because [^{35}S]methionine was included in the translation reactions two pieces of X-ray film were placed over the dried gel (the first film to block ^{35}S). The film farthest from the gel is shown.

that ZF87 binds specifically and with higher affinity to the ME1a1 element in the c-*myc* promoter than to the ME1a2 element. This finding is consistent with the results presented elsewhere that the protein that binds to the ME1a2 element is identical with or very similar to the protein that binds to the ME1a1 element (Moberg et al., 1992). However, we do not know if the HeLa cell factor that recognizes both ME1a1 and ME1a2 binds with different affinities. While we could demonstrate binding of ZF87 to the ME1a2 element by Southwestern blot hybridization, we could not demonstrate binding by EMSA. This may be due to the fact that the clone is not full length and that the amino-terminal portion of the protein may be essential for high-affinity binding to the ME1a2 site. It is also possible that HeLa ZF87 undergoes some modification that is important for binding specifically to the ME1a2 site and that such a modification does not occur in bacteria or after in vitro translation. While further experiments will be needed to address this issue, including isolation of the full-length clone, it is clear that our cloned ZF87 binds specifically to the ME1a1 element but with lower affinity to the ME1a2 element.

The amino acid sequence derived from the DNA sequence reveals a number of possible structural features. The most prominent is the presence of the six zinc-finger domains, of the Cys_2-His_2 type, in the carboxy-terminal half of the protein. This type of structure is common for a number of DNA binding proteins such as transcription factor Sp1 (Kadonaga et al., 1987), TFIIIA (Miller et al., 1985), *kruppel* (Rosenberg et al., 1986) and *serendipity* (Vincent et al., 1985). From the properties of known zinc-finger proteins, it is highly likely that the zinc fingers in ZF87 are required for binding to the c-*myc* promoter.

The first five zinc fingers in ZF87 bear the most resemblance to the zinc-finger consensus (Evans & Hollenberg, 1988). This includes the Phe/Tyr at the NH_2 side and the Phe and Leu in the center. An interesting feature of zinc fingers three and four is the Arg-X-Asp at the center (marked with asterisks in Figure 2A). These identically match the Arg-X-Asp in one of the zinc fingers of the Zif268 DNA binding protein, where the Arg has been shown to interact with a guanine in the Zif268 binding site (Pavletich & Pabo, 1991). Asp is required to stabilize the interaction (Pavletich & Pabo, 1991). Given the high frequency of guanine residues in both the ME1a1 and ME1a2 binding sites, it is probable that these arginine residues in the third and fourth zinc fingers of ZF87 will play the same role in contacting DNA.

A second interesting feature of ZF87 is the proline-rich region at the amino terminus. The first 167 amino acids are 24% proline. Of the known proline-rich proteins, the CCAAT transcription factor (CTF) (Mermod et al., 1989) and the AP-2 transcription factor (Williams & Tjian, 1991) have been shown to contain proline-rich domains that are essential for transcriptional activation. We found that certain stretches of proline and flanking amino acids did align between CTF and ZF87 (data not shown); however, there were no extended regions of homology (i.e., greater than eight amino acids). If transcriptional activation by a proline-rich domain merely requires the abundance of proline at regular intervals, then this region of the protein in ZF87 may specify a transcriptional activation domain.

A third interesting feature of ZF87 is the polyalanine tracts. These alanine tracts are predicted to form an α-helix structure with high probability (by Chou/Fasman and Robson/Garnier predictions). As outlined in Figure 2B, these tracts exist in the *Drosophila* proteins *even-skipped*, *engrailed*, *notch*, and *runt*, in the Epstein–Barr virus protein BPLF1, in the snRNP B/B′ and N-*myc* proteins, in ribosomal phosphoprotein P0, and in an antifreeze protein. A common feature of some of these proteins is that they interact with nucleic acid, indicating that this polyalanine tract may play a role in such an interaction. However, since the *notch* protein is a cell-surface factor that is important for cell–cell communication and this tract is hydrophobic, it is unlikely that it is involved directly in DNA binding. It may, for example, play a role in determining protein conformation or in protein–protein interaction; however, its function is not yet known. There is some evidence to suggest that these tracts may function as transcriptional repressors (Licht et al., 1990).

With the cloning of ZF87 it is now essential that its role in transcriptional regulation be analyzed in detail. These studies are now in progress. It is hoped that the results of these studies will lead to a better understanding of the complex control of c-*myc* expression and cell-growth control in general.

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