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Sequence-specific DNA binding by Myc proteins

(oncogene/basic motif/helix–loop–helix motif/leucine repeat/transcription factor)

EUGEN KERKHOFF*, KLAUS BISTER*†, AND KARL-HEINZ KLEMPNAUER‡

*Institute of Biochemistry, Medical School, University of Cologne, D-5000 Cologne 41, Federal Republic of Germany; and ‡Hans Spemann Laboratories, Max Planck Institute of Immunobiology, D-7800 Freiburg, Federal Republic of Germany

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ABSTRACT **Myc proteins have a tripartite carboxyl-terminal domain containing specific amino acid sequence motifs: a basic motif, a helix–loop–helix motif, and a leucine heptad repeat. Similar sequence motifs have been identified in several eukaryotic transcription factors and were shown to facilitate protein–DNA and protein–protein interactions. By using recombinant v-Myc proteins obtained by bacterial expression of full-length or partially deleted avian v-*myc* alleles, the functional relevance of these sequence motifs for Myc protein oligomerization and for DNA binding was investigated. All recombinant v-Myc proteins that have retained the carboxyl-terminal domain dimerize and specifically bind to double-stranded DNA containing the palindromic core sequence CACGTG. This and a closely related DNA sequence element have been defined previously as part of the binding sites for human transcription factors USF and TFE3, which specifically bind to the adenovirus major late promoter or the μE3 motif within the immunoglobulin heavy-chain enhancer, respectively. It is shown that a 61-amino-acid peptide sequence containing only the bipartite basic motif/helix–loop–helix domain of Myc is necessary and sufficient for dimerization and sequence-specific DNA binding of v-Myc recombinant proteins.**

Specific protein products (Myc) and nucleic acid sequences of the *myc* oncogene were originally discovered for the transduced allele in the genome of the avian retrovirus MC29 (1, 2). Subsequently, homologous v-*myc* alleles in three other avian acute leukemia viruses (CMII, OK10, and MH2) were isolated, and their common progenitor, the chicken c-*myc* gene, was identified (3, 4). The biochemical function of Myc nuclear phosphoproteins in normal or transformed cells has remained elusive. Accordingly, a functional classification of *myc* has not yet been feasible, whereas other oncogenes (*jun*, *fos*, and *myb*) specifying nuclear protein products could be classified as transcriptional activator genes (5, 6). The amino acid sequences of Myc proteins contain several motifs that may be functionally relevant (7). They were defined by homology to analogous motifs in regulatory proteins of known function, like the muscle determination factor MyoD (8–10), immunoglobulin enhancer binding proteins E12/E47 (11, 12), or Jun and Fos, two components of the transcription factor AP-1 (5). For these proteins, basic motifs, amphipathic helix–loop–helix (HLH) motifs, and leucine heptad repeats have been implicated in protein–DNA and protein–protein interactions (5, 7–13). MyoD, E12/E47, Jun, and Fos contain tandem combinations of two of these motifs in their amino acid sequences: a basic region as a putative DNA contact surface and either a HLH motif or a leucine repeat as a presumed protein dimerization domain. In Myc proteins, a combination of all three of these sequence motifs is located at the carboxyl-terminal end (7). Consistently, the Gag–Myc hybrid protein encoded by MC29 (1) had been shown to occur in monomeric and dimeric forms in transformed avian cells (14), and recombinant human c-Myc proteins were reported to form higher-order oligomeric complexes (15). Furthermore, mutational analyses of recombinant and virally encoded avian Myc proteins have revealed that the Myc carboxyl-terminal domain is necessary and sufficient for protein dimerization and nonspecific *in vitro* DNA binding (16, 17). Recently, a human transcription factor (transcription factor E3; TFE3) has been described that has a domain topography strikingly similar to that of Myc (18). TFE3 binds specifically to the μE3 motif within the immunoglobulin heavy-chain enhancer and to a site within the adenovirus major late promoter (18). The latter had been defined previously as a specific binding site for the human gene-specific upstream stimulatory transcription factor (USF), which is required for maximum expression of the major late promoter of adenovirus (19–21).

Here we show that Myc proteins are sequence-specific DNA-binding proteins sharing specificity for the DNA target sequence with human transcription factors TFE3 and USF. We also show that a 61-amino-acid peptide sequence containing only the basic and HLH motifs of Myc proteins is necessary and sufficient for protein dimerization and sequence-specific DNA binding.

MATERIALS AND METHODS

Recombinant v-Myc Proteins. The production of recombinant v-Myc proteins p59, p53, p42, and p16 has been described (17). The v-Myc recombinant protein p15 was generated by cloning a 234-nucleotide *Sau*3A fragment from the 3′ end of the v-*myc* coding region of cloned MC29 proviral DNA (16, 17) into the unique *Bam*HI site of the pET-3c expression vector and introducing the recombinant clone into *Escherichia coli* strain BL21(DE3) carrying an inducible T7 RNA polymerase gene (22).

Electrophoretic Mobility Shift Assay. Oligonucleotides containing a USF binding site (19, 20) or mutant derivatives thereof and their corresponding complementary strands generating single-nucleotide overhangs upon annealing were synthesized on an Applied Biosystems 391 DNA synthesizer. Annealed oligonucleotides were labeled to a specific activity of 5×10^6 cpm/μg by using [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase. Mobility shift assays using these probes and renatured recombinant v-Myc proteins were carried out essentially as described for the analysis of recombinant v-Myb proteins (23).

RESULTS

Purification and Oligomerization of v-Myc Recombinant Proteins. A schematic diagram of the proteins and nucleic

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Abbreviations: HLH, helix–loop–helix; USF, upstream stimulatory factor; TFE3, transcription factor E3.

†To whom reprint requests should be addressed.

acids (see below) used in this study is shown in Fig. 1. In addition to full or various partial complements of authentic v-Myc amino acid sequences, all recombinant v-Myc proteins contain at their amino termini 12 amino acids encoded by the pET-3b/c vectors (17, 22); in the case of p59, p42, and p53 these are followed by 11 amino acids encoded by *gag* and 9 amino acids encoded by v-*myc* sequences that were derived from noncoding c-*myc* sequences. The recombinant proteins p59, p42, and p16 share the authentic Myc carboxyl terminus with the c-Myc protein, whereas p53 and p15 contain 20 amino acids at their carboxyl termini that are encoded by the terminator region of the expression vectors (17, 22). All recombinant v-Myc proteins were obtained largely in the insoluble fraction of bacterial extracts, greatly facilitating their purification. Sequential detergent washes of the bacterial inclusion bodies and solubilization of recombinant proteins in 5 M urea (17) yielded large amounts of highly enriched (p59, p53, and p42) or nearly pure (p16 and p15) recombinant protein preparations (Fig. 2*A*). In immunoblotting experiments, p59, p42, and p16 reacted strongly with a polyclonal antiserum directed against purified v-Myc recombinant protein p16, whereas p15 and p53 reacted weakly. This pattern was apparently due to a high titer of antibodies in the anti-p16 serum directed against the extreme carboxyl-terminal Myc protein sequences and to a low titer of antibodies directed against the vector-encoded amino terminus of p16 (see Fig. 1*A*). As expected from the design of the recombinant proteins, p59, p42, and p16 but not p15 or p53 were recognized by antibodies directed against a synthetic peptide representing the Myc carboxyl terminus (24).

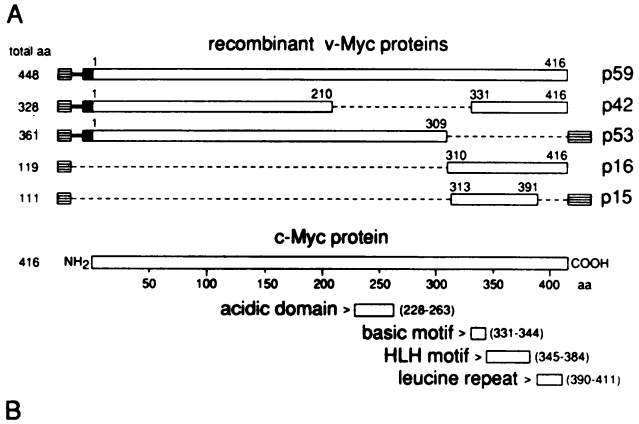

FIG. 1. Myc protein structure and USF binding site. (*A*) The structures of recombinant v-Myc proteins and of the chicken c-Myc protein are compared. Open boxes represent regions of the recombinant proteins that are encoded by v-*myc* sequences derived from the authentic c-*myc* coding region, and the amino acid numbering above these regions refers to the corresponding positions in the c-Myc protein sequence. Recombinant protein regions encoded by the bacterial expression vectors pET-3b/c, by viral *gag* sequences, or by v-*myc* sequences derived from noncoding c-*myc* sequences are indicated by hatched boxes, solid lines, or black boxes, respectively. Deletions are marked by dashed lines. Total numbers of amino acids (aa) of all proteins are given at left. The sizes and locations of putative functional domains in the Myc protein sequence are indicated by open boxes below the diagram of the c-Myc protein. (*B*) Sequences of the "upper" strands of double-stranded oligonucleotides used in the mobility shift assays are shown; they contain either the authentic USF binding site (19, 20) or two mutant forms (mut1 and mut2) of it. The binding site and its remnants in the mutant oligonucleotides are boxed.

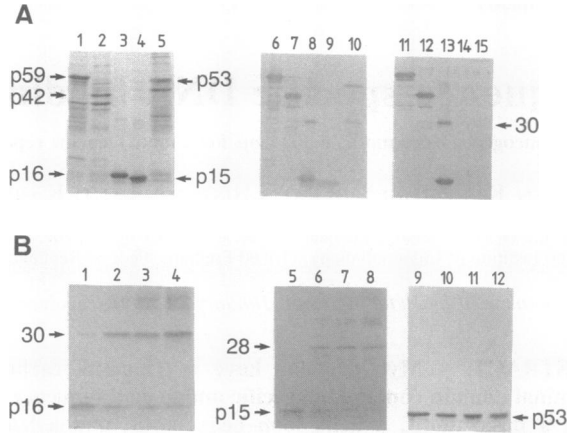

FIG. 2. Purification and chemical cross-linking of recombinant v-Myc proteins. (*A*) SDS/PAGE (12%) of urea-solubilized proteins from washed inclusion bodies (17) derived from bacterial cultures expressing v-Myc recombinant proteins p59 (lanes 1, 6, and 11), p42 (lanes 2, 7, and 12), p16 (lanes 3, 8, and 13), p15 (lanes 4, 9, and 14), or p53 (lanes 5, 10, and 15). Proteins were visualized by Coomassie brilliant blue staining (lanes 1–5) or by immunoblotting using polyclonal antibodies directed against the p16 v-Myc recombinant protein (see text; lanes 6–10) or against a synthetic peptide representing the c-Myc carboxyl terminus (ref. 24; lanes 11–15). (*B*) SDS/PAGE (12%, lanes 1–8; 7.5%, lanes 9–12) and immunoblotting of electrophoretically purified v-Myc recombinant proteins p16 (lanes 1–4), p15 (lanes 5–8), and p53 (lanes 9–12) before (lanes 1, 5, and 9) and after incubation with 0.5 mM glutaraldehyde for 20, 40, and 60 min (lanes 2–4); 10, 20, and 30 min (lanes 6–8); or 30, 60, and 90 min (lanes 10–12). For immunoblotting, polyclonal antibodies directed against the Myc carboxyl terminus (lanes 1–4) or against p16 (lanes 5–8) or monoclonal antibodies directed against an amino-terminal Myc domain (ref. 17; lanes 9–12) were used. The positions of the M_r 28,000 and 30,000 bands are indicated by 28 and 30.

We have shown previously that urea-solubilized v-Myc recombinant protein p16 forms very stable putative dimeric complexes of M_r 30,000, of which a fraction is even resistant to the conditions of SDS/PAGE (17). Notably, preparations of urea-solubilized p15 also contain an additional protein species similar in amount to, but migrating slightly faster than, the M_r 30,000 dimer in the p16 preparations (Fig. 2*A*, faint bands in lanes 4 and 9). To prove directly whether p15 also occurs in a dimeric form, electrophoretically purified and renatured recombinant v-Myc proteins p16, p15, and p53 were subjected to chemical cross-linking with diluted glutaraldehyde (17). The data in Fig. 2*B* show that p16, which contains the HLH motif and the leucine repeat, and also p15, which lacks the leucine repeat but retains the HLH motif (Fig. 1*A*), occur in solution mainly as putative dimeric complexes of M_r 30,000 and 28,000, respectively, and possibly also as higher-order oligomeric complexes. In contrast, p53 occurs in solution solely in a monomeric form (Fig. 2*B*).

Sequence-Specific DNA Binding by v-Myc Recombinant Proteins. To test whether the structural similarity between Myc proteins and the human transcription factor TFE3 (18) extends to any functional properties, mobility shift experiments were performed by using renatured preparations of the recombinant v-Myc proteins described above and a synthetic oligonucleotide (Fig. 1*B*) containing the USF binding site from the adenovirus major late promoter (19, 20). All v-Myc recombinant protein preparations, except the one containing p53, formed specific protein–DNA complexes with this oligonucleotide (Fig. 3*A*). The relative mobilities of the protein–DNA complexes formed by the v-Myc protein preparations were significantly lower than those of complexes formed between v-Myb recombinant proteins of similar size and an oligonucleotide containing a Myb binding site (Fig. 3*A*).

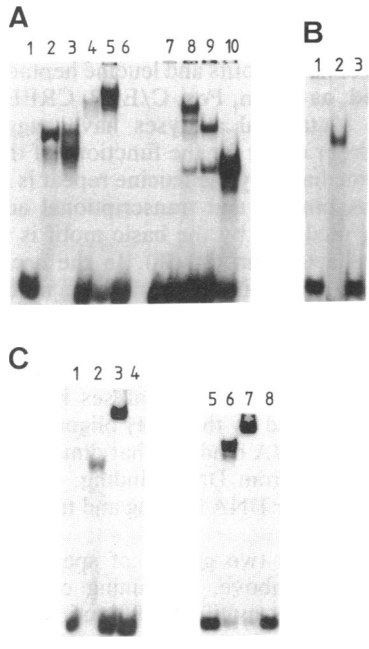

FIG. 3. Binding of recombinant v-Myc proteins to the USF binding site. (*A*) Mobility shift assays using a ^{32}P-labeled oligonucleotide containing the USF binding site. The probe was incubated prior to electrophoresis without protein added (lane 1) or with solubilized and renatured proteins from washed inclusion bodies containing v-Myc recombinant proteins p15 (lane 2), p16 (lane 3), p42 (lane 4), p59 (lane 5), or p53 (lane 6). For size comparisons, shift assays using an oligonucleotide containing a Myb binding site (6, 23) were run on the same gel. The probe was incubated prior to electrophoresis without protein added (lane 7) or with v-Myb recombinant proteins of M_r 45,000 (lane 8), 30,000 (lane 9), or 23,000 (lane 10) (23). (*B*) Mobility shift assays using the oligonucleotide containing the USF binding site without protein added (lane 1), with electrophoretically purified v-Myc recombinant protein p16 (lane 2), or with the gel-eluted M_r 16,000 fraction of renatured proteins from inclusion bodies containing v-Myc recombinant protein p59 (lane 3). (*C*) Mobility shift assays using the oligonucleotide containing the USF binding site, v-Myc recombinant proteins, and Myc-specific antibodies. The probe was incubated without protein added (lanes 1 and 5), with electrophoretically purified v-Myc recombinant protein p16 (lane 2), with p16 and antibodies specific for the Myc carboxyl terminus (lane 3), with the antibodies alone (lanes 4 and 8), with renatured proteins from inclusion bodies containing v-Myc recombinant protein p59 (lane 6), or with the p59 preparation and the Myc-specific antibodies (lane 7).

Direct proof of the identity of the proteins in the shift complexes was achieved by using highly purified protein preparations and immunological detection. Recombinant v-Myc protein p16 was highly purified by preparative gel electrophoresis (see Fig. 2); similarly, the M_r 16,000 fraction of proteins from washed inclusion bodies containing recombinant protein p59 was eluted from a corresponding gel position. Only the purified p16 recombinant protein, but not the control preparation, formed a specific complex with the oligonucleotide containing the USF binding site (Fig. 3*B*). Notably, only the slower migrating complex of the two shift complexes observed with crude p16 preparations (Fig. 3*A*) appeared when highly purified p16 was used in the shift assay (Fig. 3*B*). When antibodies directed against a synthetic peptide representing the very carboxyl-terminal Myc amino acid sequences were included in shift assays of v-Myc recombinant proteins p16 and p59, a further specific retardation of the shift complexes was observed, directly proving the presence of Myc-related proteins in these complexes (Fig. 3*C*). Taken together, the data in Fig. 3 show that v-Myc recombinant proteins can form specific protein–DNA complexes with an oligonucleotide containing a USF binding site. Furthermore, it can be directly deduced from the structure of the recombinant proteins that the 61-amino-acid peptide sequence extending from position 331 to position 391 of the avian Myc amino acid sequence and encompassing the basic and HLH motifs (Fig. 1*A*) is both necessary and sufficient for the specific protein–DNA interactions.

To investigate whether the interaction of v-Myc recombinant proteins with DNA is indeed specific for the USF binding site, mobility shift assays were carried out by using synthetic oligonucleotides containing mutations within the core sequence (CACGTG) of the USF binding site (Fig. 1*B*). Mutations or deletions within this sequence were shown previously to abolish binding both by USF and TFE3 (18, 20). Mutations in this sequence also drastically reduce binding efficiency by v-Myc recombinant protein p59, although some residual binding is observed, particularly with mutant oligonucleotide mut1 (Fig. 4*A*). The specificity of protein–DNA interactions is further indicated by comparative reciprocal mobility shift assays using v-Myc or v-Myb recombinant proteins and oligonucleotides containing USF or Myb binding sites. Specific shift complexes were formed only between v-Myc and the USF binding site or between v-Myb and the Myb binding site, respectively (Fig. 4*B*).

We have shown here (Fig. 2*B*) and previously (17) that v-Myc recombinant proteins form highly stable dimeric complexes. Furthermore, the relative mobilities of the shift complexes formed between the various v-Myc recombinant proteins and the USF binding site were significantly lower than expected from the molecular weight of the proteins used. For instance, shift complexes formed with v-Myc proteins p15 or p16 migrated with a mobility similar to that of a shift complex formed by a v-Myb protein of M_r 30,000 and much slower than a complex formed by a v-Myb protein of M_r 23,000 (Fig. 3*A*). Since Myb proteins bind as monomers to their specific binding site (25), these observations indicate that v-Myc recombinant proteins bind as oligomers, probably dimers, to the USF binding site. This was strongly supported by mobility shift assays using mixed protein preparations. When renatured recombinant proteins p15 and p59 were mixed prior to incubation with the oligonucleotide containing the USF binding site, a shift complex of intermediate mobility was formed that was not observed when either of the two proteins was used alone (Fig. 5). This is most easily explained

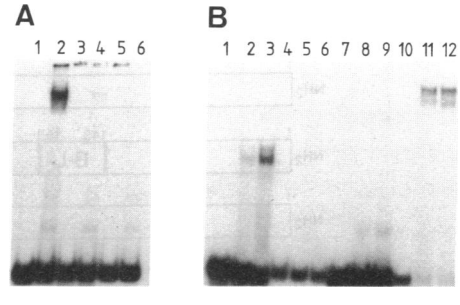

FIG. 4. Specificity of DNA binding by v-Myc recombinant proteins. (*A*) Mobility shift assays using the oligonucleotide containing the USF binding site (lanes 1 and 2), the mut1 oligonucleotide (lanes 3 and 4), or the mut2 oligonucleotide (lanes 5 and 6). The probes were incubated without protein added (lanes 1, 3, and 5) or with renatured proteins from inclusion bodies containing v-Myc recombinant protein p59 (lanes 2, 4, and 6). (*B*) Mobility shift assays using oligonucleotides containing a Myb binding site (lanes 1–3 and 7–9) or the USF binding site (lanes 4–6 and 10–12). The probes were incubated without protein added (lanes 1, 4, 7, and 10), with different amounts of the M_r 23,000 v-Myb recombinant protein (lanes 2, 3, 5, and 6), or with different amounts of renatured proteins from inclusion bodies containing v-Myc recombinant protein p59 (lanes 8, 9, 11, and 12).

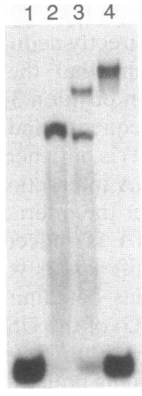

FIG. 5. Dimerization and DNA binding of v-Myc proteins. Mobility shift assays are shown using the oligonucleotide containing the USF binding site without protein added (lane 1), with renatured proteins from inclusion bodies containing v-Myc recombinant proteins p15 (lane 2) or p59 (lane 4), or with a mixture of the two protein preparations (lane 3).

by the assumption that this shift complex contains a heterodimer of p15 and p59.

DISCUSSION

Structural and now also functional properties of Myc proteins place them into a growing class of sequence-specific DNA-binding proteins implicated in the regulation of gene expression. All of these regulatory proteins identified in birds, mammals, yeast, and plants share a principal structural element—i.e., the juxtaposition of conserved amino acid sequence motifs implicated in protein–DNA or protein–protein interactions (Fig. 6). Within this class of regulatory proteins, two groups could be distinguished so far on the basis of their structural design. In the first group, tandem combinations of basic motifs and leucine heptad repeats have been identified, as in Jun, Fos, C/EBP, CREB, GCN4, and O2 (Fig. 6). Mutational analyses have suggested that a hierarchical order exists for the functions of these proteins: dimerization mediated by the leucine repeat is a prerequisite for both DNA binding and transcriptional activation, and DNA binding mediated by the basic motif is necessary for transcriptional activation (34–38). In the second group of proteins, tandem combinations of basic motifs and HLH motifs have been identified, as in MyoD and E12/E47 (Fig. 6) or in proteins encoded by the *Drosophila* cell type determination genes daughterless, hairy, twist, and achaete-scute T4/T5 (7). Again, mutational analyses have revealed that dimerization mediated by the HLH oligomerization motif is a prerequisite for DNA binding, that dimerization can function independently from DNA binding, and that the basic motif is necessary for DNA binding and trans-activation (10, 11).

In contrast to the two groups of specific DNA-binding proteins described above, containing either basic motif/leucine repeat or basic motif/HLH functional domains, Myc, USF, and TFE3 contain all three sequence motifs in a tripartite structure, basic motif/HLH/leucine repeat (Fig. 6), and hence may define a third class of basic motif DNA-binding proteins. This is also suggested by the inability of Myc proteins to form heterodimers with basic motif/HLH proteins, which readily form functional homo- and heterodimeric complexes among themselves (10, 12), or by the inability of Myc basic motif/HLH sequences to substitute for the analogous sequences in MyoD (10). Possibly, both oligomerization motifs in Myc proteins contribute to the formation of functional Myc dimers *in vivo*, although our data indicate that the bipartite basic motif/HLH domain is suffi-

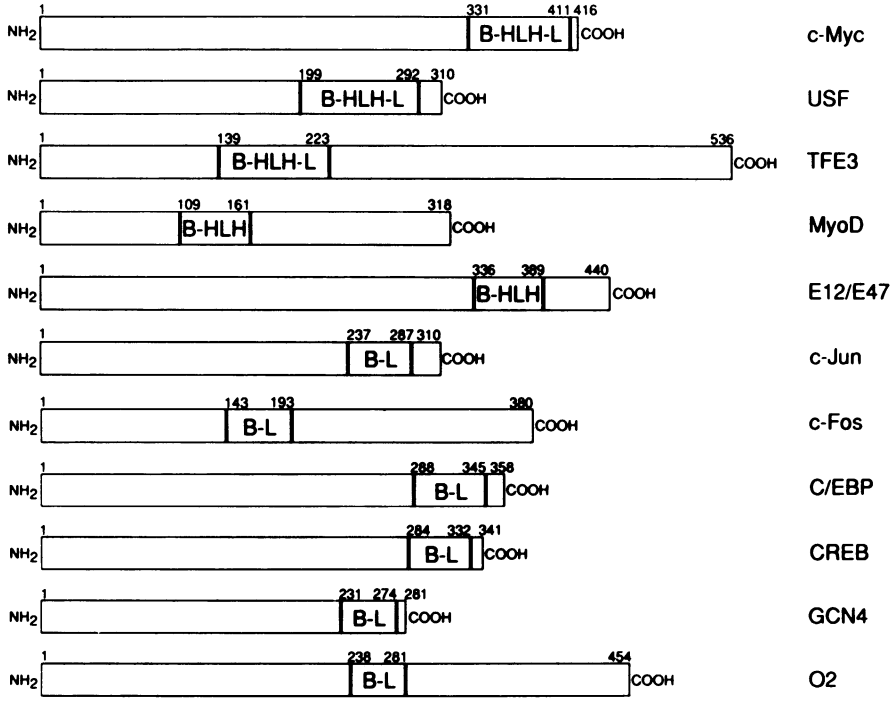

FIG. 6. Structures of regulatory proteins of birds, mammals, yeast, and plants containing conserved domains implicated in protein–DNA and protein–protein interactions. The locations of basic motifs (B), HLH motifs, and leucine heptad repeats (L) are shown schematically for the following proteins (references refer to the origins of the deduced amino acid sequences): chicken oncogene product c-Myc (26), human transcription factor USF (27), human μE3-binding protein TFE3 (18), mouse muscle determination factor MyoD (8), human κE2-binding proteins E12 and E47 (11), chicken transcriptional activator c-Jun (28), mouse transcriptional activator c-Fos (29), rat enhancer binding protein C/EBP (30), rat cyclic AMP response element binding protein CREB (31), yeast transcriptional activator GCN4 (32), and the maize endosperm-specific transcriptional activator O2 (33).

cient and that the leucine repeat is not necessary for the formation of putative dimeric forms of recombinant v-Myc proteins with sequence-specific DNA-binding activity. Alternatively, the Myc leucine repeat may be necessary for the formation of higher-order Myc oligomers or for the interaction of Myc dimers with heterologous proteins required for modulating protein–DNA interactions or for facilitating the as yet undefined putative transcriptional regulatory function of the Myc protein.

The core sequence (CACGTG) within the USF-binding site of the adenovirus major late promoter (19, 20), recognized also by TFE3 (18) and Myc (see above), is related to the core sequences within the binding sites for other regulatory proteins containing basic motif/HLH domains. These include the binding sites for TFE3 (CATGTG) within the μE3 motif of the immunoglobulin heavy-chain enhancer (18), for E12/E47 (CAGGTG) within the κE2 motif of the immunoglobulin κ chain enhancer (11), or for MyoD (CACCTG) within the muscle creatine kinase enhancer (9, 10). Similar core sequences (CATCTG) were also identified in regulatory elements within the insulin enhancer (39). All of these sequence elements conform to the consensus sequence CANNTG first defined for factor-binding elements (E boxes) within immunoglobulin enhancers (11, 40). The fact that Myc, USF, and TFE3 recognize the same specific DNA sequence element *in vitro* does not necessarily imply that these proteins have related functions *in vivo*. For instance, USF is necessary for maximum expression of the adenovirus major late promoter (19–21), but TFE3 is unable to activate transcription from it (18). Hence, transcriptional regulators with related DNA sequence specificities may well modulate expression of distinct genes, possibly depending on the genetic context of the sequence element and/or on additional interacting protein factors. The recognition of sequence-specific DNA binding by Myc proteins may facilitate the complete definition of their biochemical function and the identification of possible Myc target genes.

While this paper was under review, Blackwell *et al.* (41) and Prendergast and Ziff (42) reported that a glutathione *S*-transferase hybrid protein containing a carboxyl-terminal fragment of human c-Myc or an E12 chimeric protein containing the c-Myc basic motif bound specifically to DNA containing the same CACGTG core sequence reported here. Furthermore, Gregor *et al.* (27) reported the deduced amino acid sequence of USF, showing that its domain topography is strikingly similar to that of Myc and TFE3. The schematic structure of USF is included in Fig. 6.

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