of four cells, 100 nM GnRH). Thus, these responses require a GTP-binding protein.

IP_3 also participates in the signaling pathway activated by GnRH. Persistent current oscillations were induced in the absence of GnRH when the pipette contained 16 to 20 μM of the D isomer of IP_3 (n = 20) (Fig. 3B), but not by 100 to 200 μM of the much less active (*21*) L isomer (n = 3). Cells dialyzed with the L isomer responded to subsequent GnRH stimulation normally. However, GnRH (1 nM) induced no current oscillation when the pipette contained the competitive IP_3-receptor antagonist (*22*) heparin (150 to 300 μM) (Fig. 3C); this block could be overcome with 100 nM GnRH (n = 6).

The opening of SK channels appeared to require a rise of the concentration of intracellular free Ca^{2+}, because GnRH induced minimal K^+ currents (n = 6) in cells exposed to an intracellular Ca^{2+} buffer solution (Fig. 4A). Consistent with the effect of IP_3, the Ca^{2+} seems to come from intracellular stores (*1, 3, 5, 16, 20*). When Ca^{2+} was omitted from the bathing solution, GnRH- or IP_3-induced current oscillations persisted at least 5 min in 8 of 11 cells (Fig. 4B). Nevertheless, even in these cells the amplitude and frequency of oscillations were reduced, and in the other three cells current oscillations stopped altogether (Fig. 4C). Presumably the intracellular Ca^{2+} stores eventually became depleted. Restoring extracellular Ca^{2+} partially reversed these effects.

These observations show electrophysiological activity induced by the natural stimulatory releasing hormone in identified anterior pituitary cells. Our results also indicate a possible new role for Ca^{2+} oscillations in the Ca^{2+} economy of the cell. Cyclical release of Ca^{2+} from intracellular stores may promote voltage-gated entry of extracellular Ca^{2+}, which could help to replenish the intracellular stores and promote hormone secretion.

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Predicted Structural Similarities of the DNA Binding Domains of c-Myc and Endonuclease Eco RI

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The c-Myc oncoprotein belongs to a family of proteins whose DNA binding domains contain a basic region–helix-loop-helix (bHLH) motif. Systematic mutagenesis of c-Myc revealed that dimerized bHLH motifs formed a parallel four-helix bundle with the amino termini of helices 1 and 2 directed toward the inner and outer nucleotides of the DNA binding site, respectively. Both the basic region and the carboxyl-terminal end of the loop contributed to DNA binding specificity. The DNA binding domain of c-Myc may therefore be structurally similar to that of restriction endonuclease Eco RI.

A FAMILY OF DNA BINDING PROteins that includes c-Myc, E47, and TFEB is characterized by a conserved sequence referred to as the basic region–helix-loop-helix (bHLH) motif (*1*). The basic region of this motif contacts DNA, and the helices form a dimerization interface (*2*). Some of these proteins, such as c-Myc, have an additional dimerization motif called the leucine zipper (LZ) (*1, 3, 4*).

We sought to elucidate the tertiary structure of the c-Myc bHLH-LZ motif. As a first step, we defined the boundaries of its secondary structural elements (Fig. 1). Using the consensus amino acid sequence of bHLH proteins, we set the beginning of helix 1 at position 367 of c-Myc to demarcate the basic region from the downstream HLH motif. The end of helix 1 was set at position 378 of c-Myc since E12, E47, and TFEB each have a glycine corresponding to this position (*1, 4*) and glycines terminate one-third of the tabulated α-helices (*5*). The beginning of helix 2 for c-Myc was set at position 392, because the amino acids surrounding this position are not usually found at the NH_2-termini of α-helices (*5*). There are no helix-breaking residues between the beginning of helix 2 and the adjacent LZ, suggesting that these two elements may form a continuous α-helix.

DNA binding proteins that have a basic region fused to an LZ, such as the yeast transcription factor GCN4, dimerize with their LZs oriented in a parallel manner (*6*). To examine whether the LZs of bHLH-LZ proteins are also oriented parallelly, we substituted the LZ of a truncated form of c-Myc (MycPp) that contained only the bHLH-LZ domain (*7, 8*) with that of GCN4. The fusion protein (MycGL) bound DNA more efficiently than MycPp (Fig. 2), probably because the LZs of GCN4 homodimerize more readily than those of c-Myc (*9*). MycGL recognized the DNA binding site AC-CACGTGGT of c-Myc (*7, 10*). Altering the invariant (underlined) or inner nucleotides abolishes DNA binding by c-Myc (*7, 10*) and MycGL, while altering the outer nucleotides eliminates DNA binding by c-Myc (*7*), but

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                  5 6 7 8 9 0 1 2 2
                  3456789012345678901234567890123456789012345678901234567890123456
Myc               NVKRRTHNVLERQRRNELKRSFFALRDQIPELEN-NEKAPKVVILKKATAYILSVQAEEQKLISEEDLLRKRREQ
TFEB              RQKKDNHNLIERRRRFNINDRIKELGMLIPKANDLDVRWNKGTILKASVDYIRRMQKDLQKSRELENHSRRLEMT
E47               RERRMANNARERVRVRDINEAFRELGRMCQMHLKSDKAQTKLLILQQAVQVILGLEQQVRE
                  Basic Region      Helix 1          Loop          Helix 2          Leucine Zipper
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Fig. 1. Amino acid sequences of the bHLH domains of human c-Myc, TFEB, and E47 (*1, 4, 14*). Part of the LZs is also shown, with the leucines forming the heptad repeat underlined. Numbering refers to the positions of the c-Myc amino acids within the full-length human protein (*14*). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

only reduced the binding affinity of MycGL (Fig. 2). The reduced stringency in DNA binding specificity of MycGL relative to c-Myc is probably a result of its higher DNA binding affinity.

In order to demonstrate that MycGL bound DNA as a dimer, the GCN4 LZ substitution was performed within full-length c-Myc generating MycBGL (*8*). Cotranslated MycGL and MycBGL yielded three protein-DNA complexes in the electrophoretic mobility shift assay (Fig. 2), indicating that these proteins bound DNA as dimers (*11*). The GCN4 LZ substitution increased DNA binding, yet did not appear to alter the tertiary structure of the c-Myc bHLH domain, because it maintained DNA binding specificity, stoichiometry of the DNA binding complex, and transforming activity (*9*). We therefore used MycGL to further study the structure of the c-Myc bHLH domain.

To identify the amino acids of MycGL that recognized the inner nucleotides, we substituted small parts of the basic region with the corresponding regions of E47, a bHLH protein with a distinct inner nucleotide sequence specificity (*1*). Substitution of residues 357-62 (Fig. 1) did not alter specificity, but that of residues 361-7 did (Fig. 3A). The c-Myc and E47 proteins differ at positions 361, 362, 365, and 367 (Fig. 1). Single amino acid substitutions of Leu^{362} or Glu^{365} did not alter DNA binding specificity, whereas substitution of Arg^{367} abolished DNA binding. Substitution of both Leu^{362} and Arg^{367} conferred to MycGL the specificity of E47, whereas that of Glu^{365} and Arg^{367} abolished DNA binding (Fig. 3A). Arg^{367} is therefore the main determinant of inner nucleotide sequence specificity.

The DNA recognition sites of c-Myc and TFEB differ only in the outer nucleotides (*4, 7*). MycGL binds to both sites (Fig. 2). We attempted to confer to MycGL preferential DNA binding to the TFEB site by substituting segments of the basic region, the beginning of helix 1, or both with the corresponding TFEB sequences. Some substitutions maintained the DNA binding specificity of MycGL, while others allowed preferential binding to the c-Myc site (Fig. 3B). The latter substitutions may decrease the affinity of MycGL for DNA, revealing its c-Myc outer nucleotide sequence specificity. None of the substitutions conferred the TFEB binding specificity, suggesting that amino acids outside the basic region contact the outer nucleotides.

We next mutated other regions of the MycGL bHLH motif. Substitution of residues 389-94 of MycGL at the loop–helix 2 boundary (Fig. 1) by the corresponding

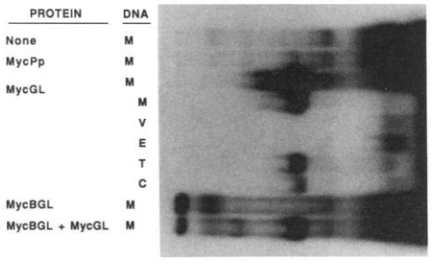

Fig. 2. DNA binding of c-Myc carrying a GCN4 LZ (*15*). Oligonucleotide M is a tandem repeat of three copies of the c-Myc binding site AC-CACGTGGT. All other nucleotides are variants of M. Oligonucleotide V has GA/TC as the invariant nucleotides; E has GC (recognized by E47) as the inner nucleotides; T and C have AT/AT (recognized by TFEB) and CT/AG as the outer nucleotides, respectively. The top three lanes and the bottom two lanes contain sixfold more ^{32}P-labeled DNA than all others. A fraction of the MycBGL-DNA complex was retained at the origin of the gel.

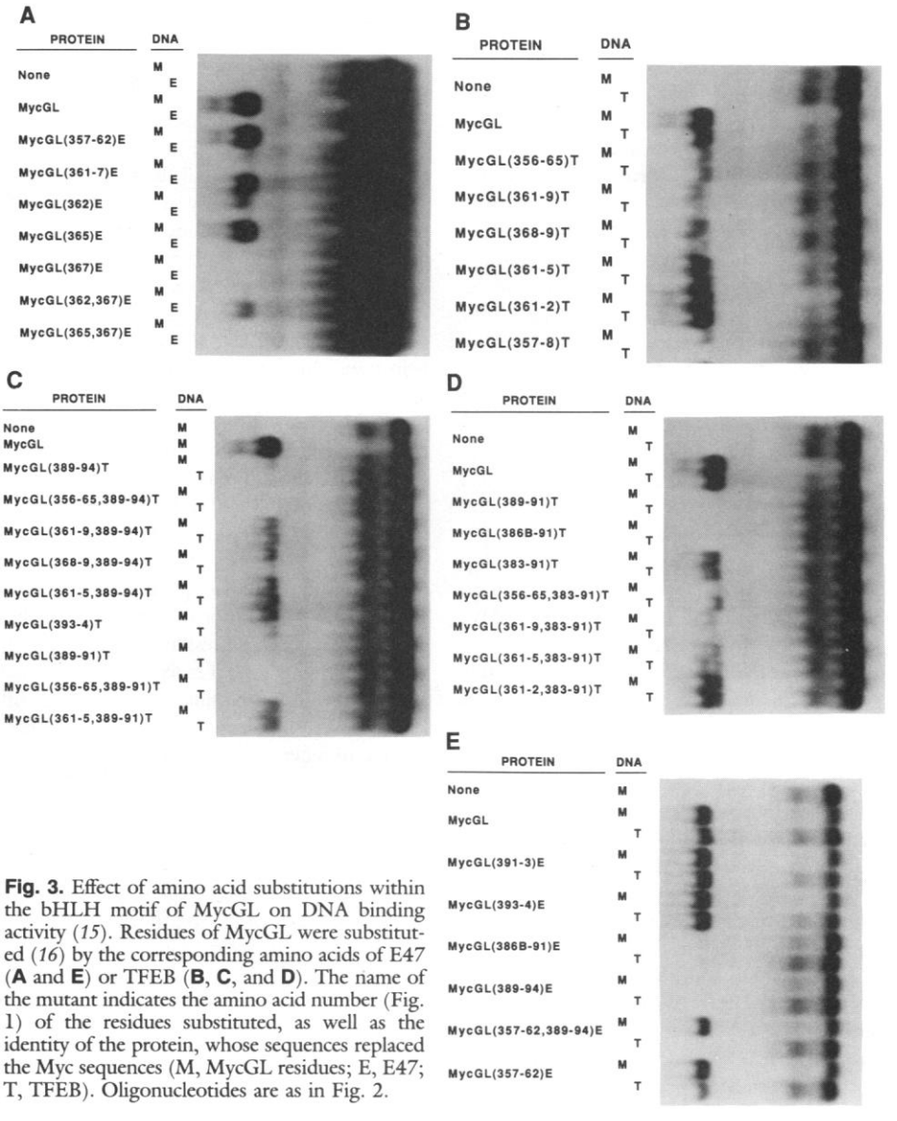

Fig. 3. Effect of amino acid substitutions within the bHLH motif of MycGL on DNA binding activity (*15*). Residues of MycGL were substituted (*16*) by the corresponding amino acids of E47 (**A** and **E**) or TFEB (**B**, **C**, and **D**). The name of the mutant indicates the amino acid number (Fig. 1) of the residues substituted, as well as the identity of the protein, whose sequences replaced the Myc sequences (M, MycGL residues; E, E47; T, TFEB). Oligonucleotides are as in Fig. 2.

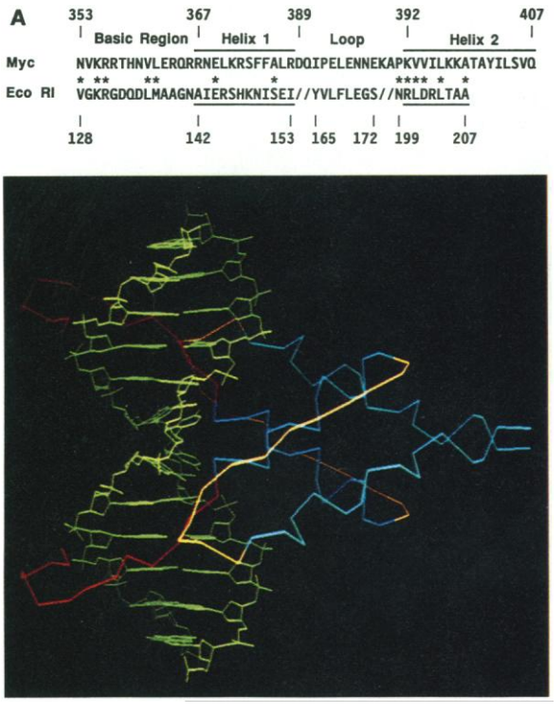

Fig. 4. Tertiary structure model of the c-Myc bHLH domain. (**A**) Alignment of the DNA recognition domains of c-Myc and Eco RI (*14, 17*). Over- and underlines indicate the extent of helices 1 and 2; asterisks amino acid identity between Eco RI and a bHLH protein. (**B**) View of the c-Myc bHLH domain (residues 353-407) in contact with a 12 nucleotide long c-Myc DNA binding site GACCACGTGGTC (green). c-Myc secondary structural elements are color-coded red, basic region; dark blue, helix 1; yellow, loop; and light blue, helix 2. Only the α-carbon atoms of the protein are shown (*18*). The five COOH-terminal amino acids of helices 2 (residues 408-412) and the LZs are not shown.

amino acids of TFEB virtually abolished DNA binding (Fig. 3C). Substituting segments of the basic region and NH_2-terminus of helix 1 with the corresponding TFEB residues restored DNA binding (Fig. 3C). Because the basic region substitutions that were done did not increase DNA binding (Fig. 3B), they must act as second-site revertants of the loop–helix 2 boundary mutation. To further characterize this interaction we made smaller substitutions. Substitution of residues 393-4 in helix 2 of MycGL did not eliminate DNA binding, whereas substitution of residues 389-91 in the loop did (Fig. 3C). The latter effect could be reversed by substituting amino acids 361-5 of the basic region with the corresponding TFEB residues (Fig. 3C). We conclude that regions specified by amino acid positions 361-5 and 389-91 of c-Myc (Fig. 1) are physically near each other.

We next increased the length of the mutated region within the loop. Substitution of residues 389-91 or 386B-91 led to loss of DNA binding, but that of residues 383-91 did not (Fig. 3D). We speculate that the position of the loop is fixed in the wild-type HLH motif, so that mutations at its COOH-terminus affect critical contacts with the basic region, DNA, or both. When a long segment of the loop is mutated, its contacts with the rest of the HLH motif may be disrupted, providing flexibility to the COOH-terminal end, so that DNA binding is not affected. The proximity of the COOH-terminal end of the loop to the basic region suggests that both structures determine outer nucleotide sequence specificity. Substitution of residues 356-65 conferred to MycGL(383-91)T the DNA binding specificity of TFEB (Fig. 3D). Neither of the individual substitutions had this effect (Fig. 3, B and D).

The physical proximity of the basic region to the COOH-terminal end of the loop was also demonstrated by substituting regions of MycGL with E47 residues (Fig. 3E).

These results suggest that the dimeric bHLH motif of c-Myc forms a parallel four-helix bundle with the NH_2-termini of helices 1 and 2 oriented toward the inner and outer nucleotides, respectively. This model is similar to the crystallographic structure of the dimeric DNA recognition domain of Eco RI (*12*). We aligned the c-Myc and Eco RI primary sequences via the NH_2-terminal amino acids of helices 1 and 2 (because the NH_2-termini face the DNA) and then assigned to the c-Myc amino acids the positional coordinates of the corresponding Eco RI residues (Fig. 4A). The COOH-terminal end of helix 1, as well as the NH_2- and COOH-terminal ends of the loop of Eco RI were not included in the alignment because of differences in length with the corresponding c-Myc elements. As a consequence, residues 379-80 and 389-90 of c-Myc were arbitrarily assigned coordinates to allow smooth transition at the helix 1–loop and loop–helix 2 junctions. Helix 2 of Eco RI is shorter than that of c-Myc, therefore residues 400-407 of c-Myc were assigned coordinates that maintained an α-helical secondary structure. To make the long axes of helices 2 parallel at their COOH-terminal ends (and thus to allow LZ dimerization) we arbitrarily introduced a bend of 18° at residue 401 of helix 2 and a second bend of 12° at residue 407.

The tertiary structure model was formed without gaps and fit all the available experimental data (Fig. 4B). The main determinant of inner nucleotide sequence specificity, Arg^{367}, is at the center of the DNA binding site. The NH_2-terminal half of the basic region and the COOH-terminal end of the loop are close to the outer nucleotides, accounting for the observation that both regions determine outer nucleotide sequence specificity. Residues 389-90 of the loop are next to residues 360-2 of the basic region, corroborating the second-site revertant mutagenesis data. In bHLH proteins the NH_2-terminal amino acid of helix 2 is a lysine or arginine; the corresponding amino acid of Eco RI, Arg^{200}, contacts the phosphate backbone of DNA (*12*). Val^{406} of helix 2 of c-Myc faces the protein interface, as expected, because it is seven amino acids away from the first leucine of the LZ. We conclude that the c-Myc bHLH-LZ domain can be modeled using the Eco RI crystal coordinates.

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16. pGEMmycGL (*8*) was linearized with Sph I and Pvu II and religated to create pGEMmycGL.SP. PCR-generated mutants (*13*) were cloned into pGEMmycGL.SP as Ppu MI–Hind III fragments.
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