furic acid. This may in part derive from the substantially higher pK_a (acidic constant) of perchloric acid (−10) relative to sulfate (−2).

In summary, we observed a complete adsorption and desorption cycle for a metal onto another metal surface with atomic resolution. In the Cu on Au(111) system, the electrolyte plays a major role in determining the structure of the upd monolayer, a result that can be explained by the degree of charge transfer between coadsorbed Cu and electrolyte. The atomic resolution obtained on close-packed metal surfaces in electrolyte with the AFM makes it a valuable technique in studying other electrochemical processes in situ.

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Methylation-Sensitive Sequence-Specific DNA Binding by the c-Myc Basic Region

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The function of the c-Myc oncoprotein and its role in cell growth control is unclear. A basic region of c-Myc is structurally related to the basic motifs of helix-loop-helix (HLH) and leucine zipper proteins, which provide sequence-specific DNA binding function. The c-Myc basic region was tested for its ability to bind DNA by attaching it to the HLH dimerization interface of the E12 enhancer binding factor. Dimers of the chimeric protein, termed E6, specifically bound an E box element (GGCCACGTGACC) recognized by other HLH proteins in a manner dependent on the integrity of the c-Myc basic motif. Methylation of the core CpG in the E box recognition site specifically inhibited binding by E6, but not by two other HLH proteins. Expression of E6 (but not an E6 DNA binding mutant) suppressed the ability of c-*myc* to cooperate with H-*ras* in a rat embryo fibroblast transformation assay, suggesting that the DNA recognition specificity of E6 is related to that of c-Myc in vivo.

THE BASIC REGIONS IMMEDIATELY NH_2-terminal to the oligomerization domains in HLH and leucine zipper proteins specifically recognize DNA with high affinity when assembled into functional dimers (*1–8*). c-Myc, a nuclear protein with unknown function, has a region rich in basic amino acids adjacent to a HLH domain (*1*), suggesting that it is a sequence-specific DNA binding protein. We sought to determine the DNA sequence recognized by the c-Myc basic region. To present the c-Myc basic region to DNA in a dimeric form, we constructed a chimeric protein that contained the dimerization interface of E12, an enhancer binding protein (*2*). The chimeric protein, termed E6, is a NH_2- and COOH-terminal truncated form of E12 (*9*), which exchanged the c-Myc basic motif for the corresponding E12 sequence (Fig. 1).

Similarity between the basic motifs of c-Myc and the putative HLH protein transcription factor E-3 (TFE-3) suggested that they might recognize similar DNA sequence elements (*10*). Therefore, a gel mobility shift assay was used to monitor binding of the E6 protein to ^{32}P-labeled oligonucleotide probes that contained either the μE3 E box site from the immunoglobulin heavy chain enhancer or the upstream stimulatory factor (USF) E box site from the adenovirus major late promoter, both of which are recognized by TFE-3 (*10*). We used TFE-3 as a positive control for binding. No complex was observed upon incubation of E6 with the μE3 E box probe ($E_{\mu E3}$); however, a strong complex was observed with the USF E box oligonucleotide probe (E_{USF}), whose formation was competed by unlabeled cognate oligonucleotide (Fig. 2A). We find that, as expected, neither intact E12 nor a truncated form of E12 that corresponded to the part of the protein found in the E6 construct bound the $E_{\mu E3}$ or E_{USF} oligonucleotide probes (*11*).

To show that E6 bound E_{USF} as a dimer through the c-Myc basic motifs, additional constructs were tested (Fig. 1). Gel fractionation of complexes formed between E_{USF} and cotranslation products of E6 and E6Δ, [a ~10-kilodalton protein that lacked 184 amino acids of the E6 NH_2-terminus] showed complexes of intermediate mobility relative to E6 or E6Δ alone (*11*). The appearance of intermediate mobility bands is consistent with the formation of E6-E6Δ dimers. In order to determine whether the integrity of the c-Myc basic motif was required for E_{USF} binding, the E6mutN, E6mutB2Q, and E6mutR mutants were generated (Fig. 1). Equivalent amounts of protein, as judged by SDS–polyacrylamide gel quantitation, were tested for binding to

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E_{USF} by an electrophoretic gel mobility shift assay. The results indicated that the former alterations reduced complex formation by 50 to 80%, while the mutR mutation eliminated binding completely (Fig. 2B). Assays with other E box oligonucleotide probes indicated that the mutN and mutB2Q constructs did not have relaxed specificity for DNA binding (*11*). These data indicate that sequences in the c-Myc basic motif determine the DNA binding capability and specificity of the E6 chimera.

To examine the ability of the c-Myc HLH-leucine zipper motifs to functionally oligomerize and to determine the effects of the c-Myc NH_2-terminus on DNA binding by the E6 c-Myc basic motif, the $EM_{B/HZ}$ and M_NE6 constructs were generated and examined by an electrophoretic gel mobility assay. $EM_{B/HZ}$ exchanges the c-Myc HLH-zipper for the E12 HLH; M_NE6 exchanges the c-Myc NH_2-terminus for that of E12, retaining the E12 HLH for oligomerization (Fig. 1). Under conditions in which E6 readily binds, we observed that $EM_{B/HZ}$, M_NE6, and full-length human c-Myc protein all failed to interact with E_{USF} (*11*). The ability of M_NE6 to dimerize was shown by its ability to be coprecipitated with antisera to MyoD (anti-MyoD) after cotranslation of MyoD and M_NE6 proteins in vitro (*11*). This raises the possibility that the c-Myc NH_2-terminus may regulate the ability of the c-Myc basic motif to contact DNA without affecting protein oligomerization (although it is also conceivable that the M_NE6 protein folds abnormally in a manner that hinders DNA binding but not protein oligomerization). Consistent with the inability of $EM_{B/HZ}$ and c-Myc to bind E_{USF}, we did not observe c-Myc homooligomers in these extracts, as detected either by glutaraldehyde crosslinking or immunoprecipitation (*11*).

To define the requirements for E_{USF} recognition by E6, a set of heterologous E box and E_{USF}-related oligonucleotides that contained dinucleotide mutations in the flanking and core regions of the CANNTG element was synthesized and used with E6 in gel mobility shift assays (Fig. 3A). Comparison of E6 with TFE-3 and USF binding activity (*12*) revealed that the c-Myc basic motif showed a more restricted repertoire of DNA binding sequences than did TFE-3 and USF. When added in ~100-fold molar excess, neither heterologous E box oligonucleotides nor E_{USF} oligonucleotides mutated at the palindromic flanks and CANNTG core region competed as well as wild-type E_{USF} for binding of E6 (Fig. 3B). In contrast, binding by TFE-3 and USF was inhibited by a variety of these mutants, indicating that TFE-3 and USF are less strict in their sequence requirements for USF E box recognition (Fig. 3B). The core CACGTG of the USF E box is apparently necessary but not sufficient for binding by the c-Myc basic motif, as several other oligonucleotides that contained this sequence but lacked the USF E box flanking residues were not bound by E6 (*11*). Taken together, we conclude that the sequence GGCCACGTGACC is recognized by the c-Myc basic motif, as presented in dimers of the E6 chimeric protein.

The observation that E6 recognized a binding site that contained a CpG dinucleotide, which in mammalian DNA often contains 5-methylcytosine, prompted us to hypothesize that a USF E box element that contained 5-methylcytosine at one or both sites of the CACGTG core might affect binding of the c-Myc basic motif. Both

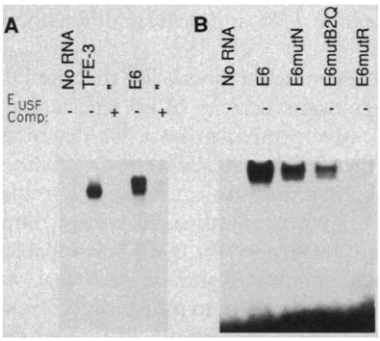

Fig. 2. c-Myc basic region–dependent binding of E6 dimers to a USF E box oligonucleotide. (**A**) The ability of E6 and TFE-3 to bind to the E_{USF} oligonucleotide was analyzed by an electrophoretic mobility shift assay (*27*). (**B**) Effects of mutations in the c-Myc basic motif of E6. The E6mutN mutant exchanges a glutamine residue for an asparagine residue that is perfectly conserved among all Myc proteins. E6mutB2Q incorporates glutamine in place of an arginine that is conserved at this position in the basic motifs of virtually all HLH proteins. The E6mutR mutant substitutes a valine for an arginine that is conserved in several HLH proteins that bind E_{USF}. Binding of E6, E6mutN, E6mutB2Q, and E6mutR was analyzed as above.

hemimethylated ($E_{USFMe\text{-}a}$ and $E_{USFMe\text{-}b}$) and fully methylated ($E_{USFMe\text{-}a/b}$) E_{USF} oligonucleotides competed poorly for E6 binding. By comparison, methylated oligonucleotides were identical to unmethylated oligonucleotides in competing for TFE-3 and USF binding activity (Fig. 3B). A titration of competing $E_{USFMe\text{-}a/b}$ oligonucleotide for binding of TFE-3 and E6 to the E_{USF} probe illustrated the effect of methylation on the binding specificity of E6 for DNA (Fig. 3C). We conclude that the c-Myc basic motif is specific for unmethylated E_{USF} sites and contacts the core CpG of E_{USF}. The CpG dinucleotide contact point, which is critical for recognition of E_{USF} by the c-Myc basic motif, is relatively rare in mammalian DNA and is a target for modification by cellular DNA methylase activity (*13*). Our observations suggest possible connections between c-Myc function and the effects of DNA methylation on gene expression and cellular differentiation (*13, 14*), if complexes that contain c-Myc recognize this site in vivo. DNA hypomethylation is correlated with expression of many genes, perhaps by allowing access to regulatory transactivators (*13*). Changes in DNA methylation are correlated with cellular differentiation in many systems in vitro and hypomethylation is a common step in the progression to neoplasia in vivo (*15, 16*). It will be important to test the premise that sites available for c-Myc binding in vivo could be controlled by DNA methylation, thereby regulating the ability of c-Myc to function at certain loci that regulate gene

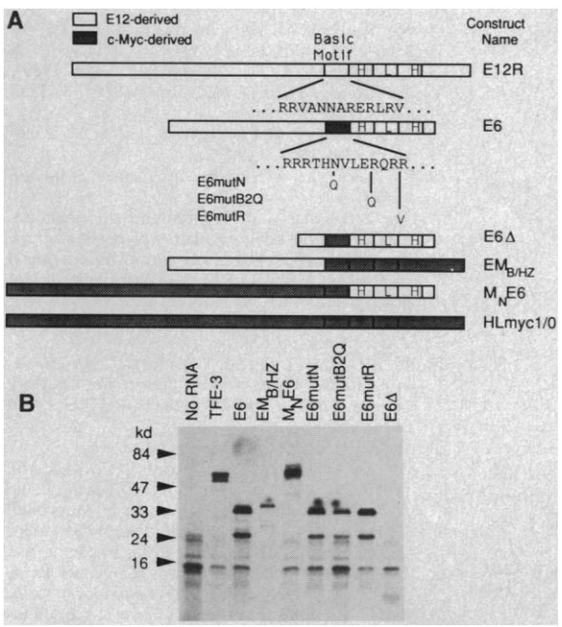

Fig. 1. Construction and expression of chimeric proteins. (**A**) E12–c-Myc chimeric constructs (*25*). (**B**) SDS–polyacrylamide gel electrophoretic analysis of ^{35}S-labeled chimeric proteins expressed in rabbit reticulocyte lysates (*26*).

expression (*17*) and cellular differentiation (*18*).

To examine the possibility that the DNA recognition specificity of E6 reflects that of the c-Myc protein in vivo, we determined whether E6 could suppress the ability of c-Myc to transform rat embryo fibroblasts (REFs) when co-expressed with a mutant H-*ras* oncogene (*19*). If E6 was capable of recognizing DNA sites in vivo that were necessary for c-Myc to transform REFs, one would predict that E6 would act in a dominant negative fashion to suppress the formation of REF foci by c-*myc* plus H-*ras*. In addition, E6 should have no effect on REF focus formation by adenovirus E1a plus H-*ras* (*20*). The results of this experiment (Table 1) show that E6 reduced the number of transformed foci several times when included with c-*myc* plus H-*ras*, but did not affect the ability of E1a to cooperate with H-*ras*. We find that expression of E6mutR, the mutant that failed to bind DNA (Fig. 2B), had no effect when included in transfections with either E1a or c-*myc* plus H-*ras*. Thus, E6 can specifically inhibit c-Myc function in *ras* cotransformation assays in a manner that correlates with the ability of E6 to recognize DNA.

These data are consistent with the interpretation that the DNA recognition specificity of E6 reflects that of the c-Myc protein in the oligomeric form in which it exists in vivo. One can rule out two other interpretations. First, it is unlikely that E6 inhibits transformation by complexing directly with the c-Myc protein because E12 cannot interact with c-Myc (*2, 11*). Second, "squelching" of c-Myc function by any potential E12-derived transcriptional activation domains in E6 can be ruled out because E6mutR has no effect on c-Myc transformation. One cannot rule out the possibility that E6 activates an endogenous gene that indirectly suppresses c-Myc function, but not E1a function in the H-*ras* cotransformation assay. However, this and other interpretations are clearly more complex than that suggested above. To assess the ability of intact c-Myc protein to bind specifically to DNA, it will be necessary to identify c-Myc dimerization partners or post-translational modifications that enable c-Myc to act in such a manner. At relatively high concentrations, homo-oligomeric interactions have been observed with bacterially produced c-Myc (*21*). Indeed, at similar concentrations bacterially expressed glutathione-*S*-transferase fusion protein that contains the c-Myc COOH-terminus binds specifically to the same CACGTG core sequence reported here (*22*). It is possible that a c-Myc homo- or heterooligomer recognizes a somewhat different sequence than that presented here. However, the observation that E6 can suppress the ability for c-Myc to cooperate with mutant H-*ras* in REF transformation assays suggests that in vivo sites required for c-Myc transformation will resemble the USF E box site.

Table 1. Rat embryo fibroblast (REF) transformation assay. Second passage REF cultures were transfected with the following plasmids and scored for foci two weeks after transfection (*23*). pT22, mutant human H-*ras*^{Val12} (*19*); LTR-Hmyc, normal human c-*myc* under the control of the Moloney murine retroviral LTR enhancer-promoter (*24*); p1A, genomic adenovirus type 5 DNA encoding the E1A region (*24*); CMV E6 and CMV E6mutR, the E6 and E6mutR E12–c-Myc chimeras under the control of the human cytomegulovirus enhancer-promoter.

Transfected DNA	Number of REF foci/plate			
Experiment number:	1	2	3	4
Mock transfected	0	0	0	—
pT22	1	0	0	—
LTR-H*myc*	0	0	0	—
CMV E6	0	0	0	—
CMV E6mutR	—	0	0	—
LTR-H*myc* + pT22	17	13	15	—
LTR-H*myc* + pT22 + CMV E6	2	1	4	4
LTR-H*myc* + pT22 + CMV E6mutR	—	12	17	23
p1A	—	—	0	0
p1A + pT22	—	—	13	15
p1A + pT22 + CMV E6	—	—	12	15
p1A + pT22 + CMV E6mutR	—	—	16	18

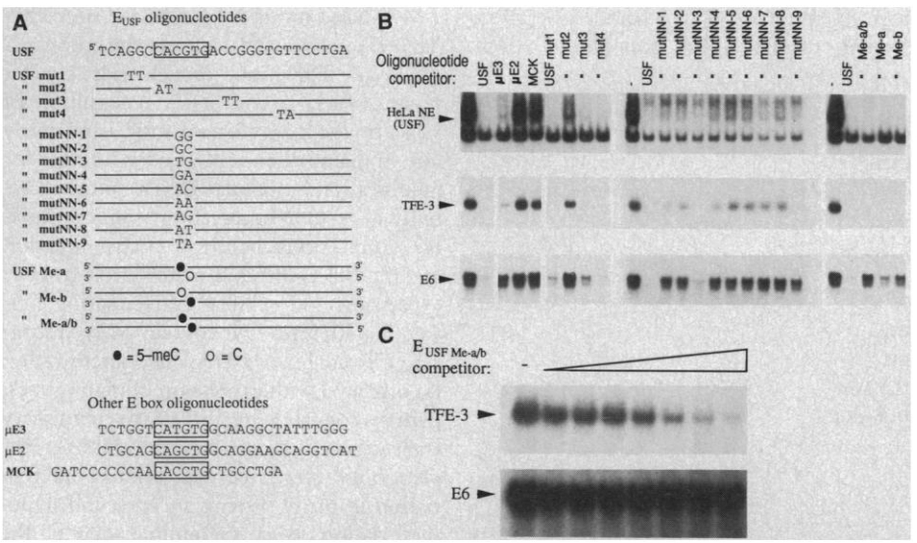

Fig. 3. Competition for USF, TFE-3, and E6 complex formation by various oligonucleotides. (**A**) Sequences of mutated or methylated oligonucleotides used for competitions in electrophoretic mobility shift assays. 5-Methylcytosine, 5-meC; cytosine, C. (**B**) Competition for binding by USF, TFE-3, and E6 to the E_{USF} oligonucleotide (*28*). (**C**) Competition by increasing amounts of $E_{USF\text{-}Me\text{-}a/b}$ oligonucleotide for binding of TFE-3 and E6 to the E_{USF} oligonucleotide (*29*). The following amounts of unlabeled methylated competitor were included per lane (~0.5 ng of probe was present in the competition): 0, 1, 2, 4, 8, 16, 32, 64 ng.

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23. Rat embryo fibroblasts (Whittaker Bioproducts) were transfected by a modified calcium phosphate method (*29*). The c-*myc* and H-*ras* plasmids (5 μg of each) and 10 μg of the E6 or E6mutR plasmids were used for transfections with the pBluescript SK+ (Stratagene) vector used as carrier DNA to bring the total plasmid DNA to 20 μg in each transfection. The human c-Myc (LTR-H*myc*), adenovirus E1a (p1A), and mutant H-*ras* (pT22) expression vectors plasmid have been described (*19, 30*). The CMV E6 and CMV E6mutR vectors were generated by moving the Eco RI–Hind III inserts from pBS+ to pcDNAI (Invitrogen), which encodes a cytomegulovirus promoter immediately upstream of the insert coding regions. Cells were divided (1:3) 2 days after transfection and fed at 4-day intervals with Dulbecco's modified Eagle's medium that contained 5% fetal bovine serum. Foci appeared 8 to 12 days after transfection and were scored at 14 days by giemsa staining.
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25. Fragments that encoded the desired amino acid sequence were amplified by the polymerase chain reaction and cloned into the pBS+ vector (Stratagene). The parental expression vector E12/5′, from which E6, the E6mut clones, E6Δ, and $EM_{B/HZ}$ were derived, was produced from a fragment that encoded amino acid residues 112 to 336 from pE12R (*2*) with the 5′ primer, 5′-GAAAGCTTACCATGGCTGGCAGCTCCGGGGATGC-3′ (E12-E) and the 3′ primer, 5′-GAGTCGACGCTCCTTCTCCCGCTCG-3′ (E12-C). E6 was generated by subcloning into E12/5′, a pE12R-derived polymerase chain reaction (PCR) fragment produced by the following primers: 5′ primer, 5′-AGGTCGACGAACACACAACGTCTTGGAGCGCCAG CGCAGGAACAAAGTGCAGGAGGCCTTG-3′ (Mbasic) and 3′ primer, 5′-GAGAATTCTTAACAGGTTCCGCTCTCGCACTTG-3′ (E12-B). The E6mut constructs were generated by subcloning into E12/5′ pE12R-derived PCR fragments produced with the 3′ primer E12-B and the mutant E6 5′ primers listed below. Mutations were introduced individually at the underlined positions noted above in the primer Mbasic: E6mutN, CAG at the first position (Asp to Gln); E6mutB2Q, CAG at the second position (Arg to Gln); E6mutR, GTG at the third position (Arg to Val). E6Δ was derived from E6 by subcloning a PCR fragment generated with the primers E12-C and E12-F, AGAAGCTTACCATGGCGGCTGACCACTCGGAGGAG [the translation initiation site in E6Δ corresponds to amino acid 296 in E12 (*2*)]. $EM_{B/HZ}$ was constructed by subcloning into the E12/5′ vector a PCR fragment that encoded amino acids 347 to 439 (c-Myc basic-HLH-zipper) from pHL*myc* 1/0 (*25*); the primer sequences were 5′ primer, 5′-AGGTCGACGAACACACAACGTCTTGG-3′ and 3′ primer, 5′-GAGAATTCAGGACATTTCTGTTAGAAG-3′. The parental expression vector 5′Hmyc from which M_NE6 was derived was produced from a fragment that encoded amino acids 1 to 346 from pHL*myc* 1/0 (*25*) with the 5′ primer, 5′-GAAAGCTTCCCCGCCGCTGCCAGGAC-3′ and 3′ primer, 5′-CAGTCGACGGACATTCTCCTCGGTGTCCG3′. M_NE6 was constructed by subcloning the Sal I–Eco RI fragment from E6 into the 5′Hmyc vector. The structure of the basic-HLH region of all chimeric constructs was verified by DNA sequencing. The TFE-3 clone pBS-λ3 (*10*) was obtained from H. Beckmann and T. Kadesch.
26. Transcription and translation reactions including [^{35}S]methionine (40 μCi) were performed as described (*3*). An aliquot (~0.5 μl) of a standard 50-μl translation reaction was analyzed by SDS–polyacrylamide gel electrophoresis and fluorography.
27. Standard binding reactions were performed as described (*2*) with the addition of a highly degenerate nonspecific single-stranded oligonucleotide (~0.6 μg). Binding reactions contained reticulocyte extract (2 μl) programmed with E6 or TFE-3 RNA and double-stranded E_{USF} probe (~0.5 ng), which was produced by annealing complementary oligonucleotides as described (*3*) and end-labeling with γ-[^{32}P]ATP. For competition, annealed unlabeled E_{USF} (~100 ng) was added prior to the addition of the extract proteins. Protein-DNA complexes were fractionated on 5% polyacrylamide gels (37.5:1, acrylamide:bis-acrylamide) as described (*2*) and processed for autoradiography.
28. The proteins in the binding reactions were derived from HeLa nuclear extract, in the case of USF (~5 μg of nuclear extract protein per reaction was used), or from programmed reticulocyte extracts (*25*) in the case of TFE-3 and E6. For methylated E_{USF} oligonucleotides, 5-methyl cytosine was incorporated at the central CpG of the recognition site GGACACGTGACC on each strand (Genosys). Hemimethylated oligonucleotides were generated by annealing either methylated strand with the complementary unmethylated strand.
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Distinct Protein Targets for Signals Acting at the c-*fos* Serum Response Element

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The c-*fos* serum response element (SRE) is a primary nuclear target for intracellular signal transduction pathways triggered by growth factors. It is the target for both protein kinase C (PKC)–dependent and –independent signals. Function of the SRE requires binding of a cellular protein, termed serum response factor (SRF). A second protein, $p62^{TCF}$, recognizes the SRE-SRF complex to form a ternary complex. A mutated SRE that bound SRF but failed to form the ternary complex selectively lost response to PKC activators, but retained response to PKC-independent signals. Thus, two different signaling pathways act through discrete nuclear targets at the SRE. At least one of these pathways functions by recruitment of a pathway-specific accessory factor ($p62^{TCF}$). These results offer a molecular mechanism to account for the biological specificity of signals that appear to act through common DNA sequence elements.

REGULATION OF CELLULAR PROLIFeration and differentiation is coordinated by extracellular signals. The receptors that transduce these signals share common structures and enzymatic activities, and activate common intracellular signal transduction systems. Yet growth factors act with a remarkable degree of specificity—they trigger unique responses in a given cell and, sometimes, different responses in different cells. Ultimately, this specificity must reside in the genome, because these responses are driven by changes in cellular gene expression.

The c-*fos* proto-oncogene is a primary genomic target for extracellular signals (*1*). Many of these signals act through a small regulatory element that flanks the c-*fos* gene, the serum response element (SRE) (*2*). The SRE is the site of action for at least two distinct signal transduction pathways—one that involves the activation of protein kinase C (PKC) and another that transmits signals by a PKC-independent mechanism (*3–8*). For the SRE to function, binding of a cellular protein termed serum response factor (SRF) is required (*9–14*). Yet the precise function of SRF in signal transduction is not clear, because there is no change in the properties of SRF isolated from stimulated cells. Indeed, the SRE remains constitutively bound by protein in cells regardless of the transcriptional state of the gene (*15*).

Two additional proteins bind to the SRE in vitro. One protein, $p62^{DBF}$ (for direct binding factor) binds directly and asymmetrically to the 5′ side of the SRE (*16*). A second protein, $p62^{TCF}$ (for ternary com-

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