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Genetics

Construction and expression of a monomeric c-Jun protein that binds and activates transcription of AP-1-responsive genes

(DNA-binding protein/transactivation)

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ABSTRACT **c-Jun is a typical member of the bZIP (basic zipper) family of dimeric transcriptional activators. These proteins contain a basic region responsible for DNA sequence recognition and a leucine zipper that mediates dimerization. bZIP proteins regulate a large number of important physiological functions and, therefore, present an interesting target for molecular interference and mimicry. As a step toward the development of peptide and nonpeptide analogs of such proteins, we constructed a derivative of c-Jun that binds DNA as a monomer. This construction was done by connecting a second basic region to the natural basic region of c-Jun by means of a short peptide loop. Although the polypeptide backbone of the second basic region has an inverted polarity relative to that of the natural basic region, the monomeric c-Jun protein binds DNA with reasonably high affinity and indistinguishable specificity from the wild-type, dimeric c-Jun protein. Furthermore, the monomeric c-Jun protein can activate transcription *in vivo*. These findings indicate that the polypeptide backbone of the basic region contributes little to sequence recognition and that the leucine zipper is not directly involved in transcriptional activation.**

Many sequence-specific transcription factors, both prokaryotic and eukaryotic, interact with DNA as preformed dimers (1–8). Two large families of dimeric eukaryotic transcription factors were recently identified: the bZIP (for basic zipper) and the HLH (helix–loop–helix) proteins (3–5, 7, 8). These proteins are involved in a variety of physiological functions, including the control of cell proliferation and differentiation and in mediating the actions of polypeptide hormones, cytokines, and growth factors. The DNA-binding domains of both families are constructed of a basic region rich in positively charged amino acids, which interacts directly with the DNA, and an adjacent dimerization motif. The bZIP dimerization motif is an amphipathic α-helix containing several heptad repeats of leucine residues, responsible for formation of a parallel coiled-coil known as the leucine zipper (3, 9, 10). In both cases, the dimerization domains mediate not only homotypic interactions but also heterotypic interactions that expand the regulatory potential of these proteins. For example, a c-Jun–c-Fos heterodimer is more stable than a c-Jun homodimer and, therefore, has higher DNA-binding activity and is a more efficient transcriptional activator (11–17). Heterodimerization of MyoD or myogenin with E12 and E47 increases their affinity to the E box sequence of muscle-specific promoters (5, 7, 8).

The localization of dimerization and DNA-binding functions of bZIP and helix–loop–helix proteins to relatively small and well-defined sequence motifs has raised the possibility of synthesizing analogs of these proteins that could interfere with either their dimerization or DNA-binding activities. Indeed, several groups have described that short synthetic peptides corresponding in sequence to the basic regions and leucine zippers of certain bZIP proteins can bind DNA *in vitro* (18, 19). We are interested in preparing analogs of c-Jun that are functional *in vivo* and could be prototypes for designing totally synthetic analogs; these synthetic analogs could eventually be used as competitive inhibitors of DNA binding. We also wanted to determine whether the leucine zipper of c-Jun is required for any other activity besides dimerization. By constructing a c-Jun protein that binds DNA as a monomer, we show that dimerization is not essential for transcriptional activation and that c-Jun can activate transcription by itself, without forming dimers with other bZIP proteins.

MATERIALS AND METHODS

Plasmids, Cell Culture, and Transfections. Construction of c-Jun, cJunΔLZ expression vectors, and the −79/+170jun–CAT, −79/+170ΔAP-1jun–CAT reporters has been described (20–22). To generate the monomeric c-Jun expression vectors, codons 278 and 279 of c-Jun in the Rous sarcoma virus–c-Jun vector (20) were mutated from GCC CGG to GCG CGC to create a *Bss*HII site. The resulting plasmid was digested by *Bss*HII and *Xho* I and ligated to phosphorylated oligonucleotides coding for the loop and a new basic region as shown in Fig. 1*C*. The exact sequences of the oligonucleotides are available upon request. To construct the truncated Jun (t-Jun) expression vector a *Pst* I–*Bam*HI fragment encoding amino acids 222–331 of c-Jun was cloned into pET-8C (23) by using the adaptor: 5′-CATGGCTAGCGAATTCCTGCA
3′-CGATCGCTTAAGG-5′.

F9 cells were grown and transfected as described (20, 21).

Expression and Purification of Recombinant Proteins. To adapt the c-Jun cDNA to the pET-8c vector (23), two nucleotides preceding its initiator ATG codon were mutated to create a *Bsp*HI site by site-directed mutagenesis. The *Bsp*HI–*Bam*HI fragment from Rous sarcoma virus–c-Jun (20) was inserted into pET-8c between the *Nco* I and *Bam*HI sites to generate pET-8c/c-Jun. To express monomeric Jun (m-Jun), the C-terminal coding region of c-Jun in pET-8c/c-Jun was replaced by the same region of m-Jun. The plasmids were transformed into *Escherichia coli* BL21(DE3)pLysS. The cells were induced, and Jun proteins were extracted from inclusion bodies and renatured as described (24). The proteins used in this report were purified to near homogeneity by heparin–agarose chromatography (24). Protein concentrations were determined by the Bradford assay (Bio-Rad). The N-terminal sequence of the recombinant c-Jun was determined by J. Woodgett (Ludwig Institute for Cancer Research) as NH_2-Thr-Ala-Lys-Met-Glu-Thr-Thr, the expected sequence after removal of the first methionine residue. Trans-

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Abbreviations: TRE, phorbol 12-myristate 13-acetate-responsive element; DSS, disuccinimidyl suberate; t-Jun, truncated Jun; m-Jun, monomeric Jun.

fection and immunoprecipitation of protein in F9 cells were done as described (25, 26).

Mobility-Shift Assay. Mobility-shift assays (27) contained the indicated amounts of the different Jun proteins, 1 ng of ^{32}P-labeled phorbol 12-myristate 13-acetate response element (TRE) probe, 100 ng of sonicated salmon sperm DNA, 12 mM Hepes·KOH (pH 8.0), 50 mM KCl, 6 mM $MgCl_2$, 1 mM EDTA, 10% (vol/vol) glycerol, 5 mM dithiothreitol, and 80 μg of bovine serum albumin in a total volume of 20 μl. After a 20-min incubation at room temperature, reaction mixtures were loaded on 5% native polyacrylamide gels (acrylamide/bisacrylamide, 40:1). Electrophoresis was done in 0.4× Tris/borate/saline (TBE; 1× TBE is 90 mM Tris/64.6 M boric acid/2.5 mM EDTA, pH 8.3) at room temperature. The mobility-shift experiments were quantitated by counting the radioactivity of the dried gels with the Ambis radioanalytic imaging system.

jun–TRE, consensus TRE (16), NF1, and Sp1 (28) oligonucleotide probes were described previously.

DNase I Footprinting and Methylation Interference. The c-*jun* promoter probe was labeled at the *Nco* I site at position −132 on the noncoding strand (20) and incubated with either c-Jun (80 ng), m-Jun (1.6 μg), or bovine serum albumin (10 μg) and digested with either 1 or 3 ng of DNase I, as described (29). For methylation interference the c-*jun* promoter fragment (−132 to +170) was labeled at position −132 either on the coding (by T4 polynucleotide kinase) or noncoding (by avian myeloblastosis virus reverse transcriptase) strands. Methylation interference was done as described (29).

Chemical and UV Cross-Linking and Sedimentation Analysis. One hundred microliters of either c-Jun or m-Jun (both at 0.07 mg/ml) were treated with either 2 μl of dimethyl sulfoxide or 2 μl of 10 mM disuccinimidyl suberate (DSS) in dimethyl sulfoxide for 10 min at room temperature. The reactions were quenched by adding 5 μl of 1 M lysine and analyzed by electrophoresis on a 10% polyacrylamide/SDS gel stained with Coomassie blue. For UV cross-linking experiments, protein–DNA complexes were allowed to form for 20 min on ice. Samples were treated with UV light (254 nm) for another 20 min on ice, 4 cm from the light source. For further cross-linking by DSS, 2 μl of 10 mM DSS was added to each sample. The mixture was incubated at room temperature for 10 min and then quenched by adding 2 μl of 1 M lysine. After this, samples were boiled in Laemmli sample buffer and analyzed on SDS/12% PAGE. The gel was dried and exposed with intensifying screen at −80°C overnight.

Two micrograms of purified c-Jun or m-Jun was mixed with protein molecular mass markers (Bio-Rad) and sedimented through a 15–60% (vol/vol) glycerol gradient in buffer Z [25 mM Hepes·KOH, pH 8.0/12.5 mM $MgCl_2$/10% (vol/vol) glycerol/0.1% Nonidet P-40 1 mM dithiothreitol] containing 100 mM KCl. After 19 hr at 50,000 rpm in an SW55.2 rotor at 20°C the gradient was fractionated, and each fraction was analyzed by SDS/PAGE, silver staining, and immunoblotting for the presence of the molecular mass markers, c-Jun and m-Jun.

RESULTS

Experimental Approach. c-Jun is a bZIP protein that is a major component of the AP-1 complex, consisting of Jun homo- and heterodimers and Jun–Fos heterodimers (30, 31). These proteins interact with a common sequence known as the AP-1 site or the TRE (30, 31). Like other bZIP proteins, the leucine zipper of c-Jun determines its ability to form homo- and heterodimers (14–17). The basic region appears unstructured before DNA binding and assumes a helical conformation after contacting its recognition site (18, 19, 32, 33). From these and other findings, Vinson *et al.* (34) proposed that upon interaction with the DNA, the basic region undergoes structural transition, allowing the protein to bind

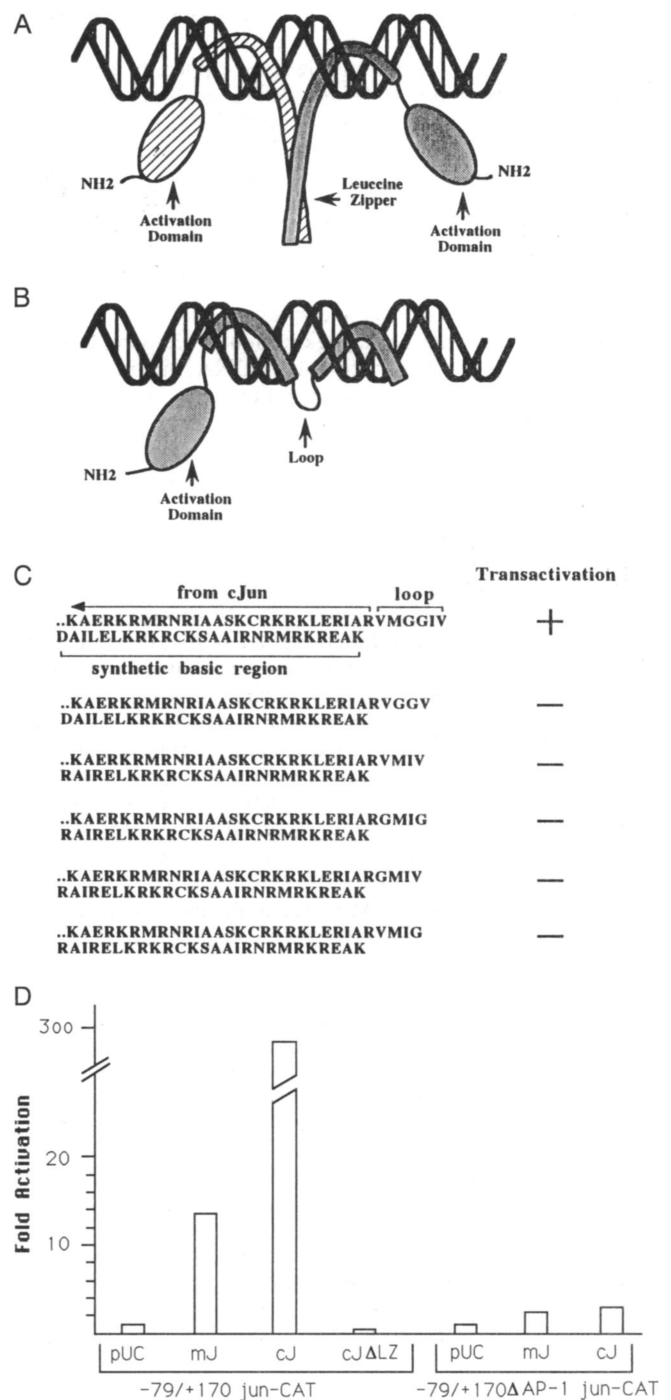

FIG. 1. Schematic representation of wild-type c-Jun (*A*), according to the scissors-grip model and monomeric c-Jun (*B*). (*C*) Primary structures of the DNA-binding domains of the monomeric c-Jun proteins using the single-letter amino acid code; the loop sequences are indicated in italics. Note that the second basic region has been modified to contain leucine and aspartate, instead of the last two arginines in the original c-Jun sequence. These arginines are not conserved among other bZIP proteins (18). In addition, the polypeptide backbone of the second basic region is in inverted polarity to that of the original basic region. Ability of the different monomeric c-Jun constructs to activate the c-*jun* promoter is indicated as positive (+) or negative (−). (*D*) Transactivation by c-Jun (cJ), m-Jun (mJ), and cJunΔLZ. F9 cells were transfected with the indicated reporters and expression vectors (2 μg of each plasmid per plate), and chloramphenicol acetyltransferase (CAT) activity was determined 24 hr later. The results are the mean of three experiments and are presented as the fold increase in acetyltransferase activity over the base line seen with cJunΔLZ.

its cognate DNA sites like a scissors grip (Fig. 1*A*). According to this model, it may be possible to link two basic regions by a peptide loop, instead of a leucine zipper, to generate a bZIP protein that binds DNA as a monomer (Fig. 1*B*). Hence, we connected a second, slightly modified, basic region to the basic region of c-Jun via the peptide-loop sequences shown in Fig. 1*C*. Glycines were included to increase loop flexibility. To allow synthesis of the protein as a single polypeptide chain, the second basic region has the same amino acid sequence as the first region, but this sequence follows the C-terminal to N-terminal direction. Despite its inverted polarity, the second basic region displays the same order of side chains as the original basic region, and if the peptide backbone itself does not participate in DNA binding, it may possess similar DNA-binding specificity. To identify a construct encoding a potentially monomeric c-Jun protein capable of functioning *in vivo*, we left the transactivation domain as part of the protein because, even though this domain is not necessary for DNA binding, it helps monitor activity of the protein. The various constructs were tested for their ability to transactivate the AP-1-responsive c-*jun* promoter (20). One construct tested was functional (Fig. 1*D*). Because this construct displayed much lower activity toward a mutated c-*jun* promoter, lacking a functional AP-1 site, this construct apparently acted in a sequence-specific manner. Immunoprecipitation analysis of transfected F9 cells indicated that the monomeric c-Jun construct expressed a protein with the predicted mobility (Fig. 2). Expression of this protein was 8-fold less efficient than expression of wild-type c-Jun, probably due to the more rapid degradation of the monomeric protein. This decreased expression could account for much of the decreased transactivation potential of the monomeric c-Jun construct.

The Designed Protein Is Monomeric Before and After DNA Binding. To further characterize its activity and physical properties, the protein encoded by this construct, m-Jun, and its wild-type counterpart, c-Jun, were expressed in *E. coli* by using the T7 expression system (23). Both proteins were extensively purified, and their aggregation state was examined by chemical cross-linking and sedimentation analysis. Treatment of c-Jun with the homobifunctional cross-linking agent (DSS) resulted in the formation of stable c-Jun dimers, whereas no cross-linking of m-Jun was seen (Fig. 3*A*). Sedimentation analysis with glycerol gradients indicated that c-Jun exists in solution as a mixture of monomers and dimers, whereas m-Jun is exclusively monomeric (Fig. 3*B*).

To demonstrate unequivocally that m-Jun binds to the TRE as a monomer, we did additional cross-linking experiments. Both c-Jun and m-Jun were incubated with a large excess of ^{32}P-labeled *jun*–TRE sufficient to saturate both proteins. These mixtures were exposed to UV irradiation to cross-link the protein molecules to DNA and DSS to cross-link protein molecules to each other. In preliminary experiments we found that neither cross-linking agent alone was sufficient for generating a composite protein–protein and protein–DNA adduct. After cross-linking, the mixtures were resolved on polyacrylamide/SDS gels, and the protein–DNA adducts were visualized by autoradiography. Fig. 3*C* shows that the c-Jun–TRE adduct migrated with an apparent molecular mass of 96 kDa, consistent with binding of a protein dimer to the TRE. However, the m-Jun–TRE adduct migrated with an apparent molecular mass of 46 kDa, consistent with binding of a protein monomer to the TRE.

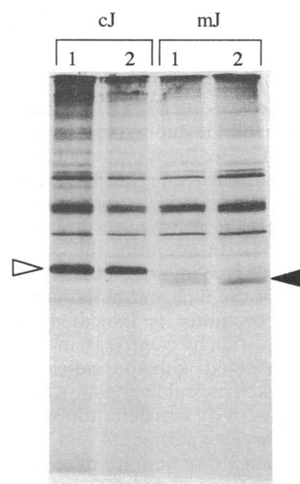

FIG. 2. Immunoprecipitation analysis of Jun protein expression. Expression vectors encoding wild-type c-Jun (cJ) and m-Jun (mJ) were transfected into F9 cells. Twelve hours after transfection the cultures were labeled for 3 hr, and ^{35}S-labeled Jun proteins were isolated by immunoprecipitation and resolved by PAGE. Migration positions of c-Jun and m-Jun are indicated by open and solid arrowheads, respectively. Two separate experiments are shown.

Monomeric c-Jun Binds DNA Specifically and Efficiently. Mobility-shift assays were done to compare the relative affinities of c-Jun and m-Jun to the *jun*–TRE (Fig. 4*A*). m-Jun was ≈one-tenth as efficient as c-Jun in binding this sequence. The complex formed by m-Jun with either the *jun*–TRE or a consensus TRE sequence had an electrophoretic mobility intermediate to those of the slower moving complex formed by wild-type c-Jun and the faster moving complex formed by a truncated c-Jun (t-Jun), consisting of its 110 C-terminal amino acids (Fig. 4*B*). These differences in electrophoretic mobility are consistent with m-Jun binding to the TRE as a monomeric 36-kDa protein, whereas c-Jun and t-Jun bind as dimeric 38-kDa and 15-kDa proteins, respectively. All three proteins bound both TRE probes with similar efficiencies, and competition experiments showed that binding of m-Jun to the *jun*–TRE was specific (Fig. 4*C*).

The specificity of m-Jun binding to DNA was further demonstrated by DNase I footprinting (Fig. 5*A*) and methylation interference (Fig. 5*B*). Both c-Jun and m-Jun generated indistinguishable protection and interference patterns centered around the TRE of the c-*jun* promoter. Interestingly, methylation of the first guanine upstream to the 5′-TGACATCA-3′ sequence fully interfered with binding of both c-Jun and m-Jun, whereas methylation of the second guanine partially interfered with their binding. Hence, both Jun proteins appear to contact these residues, even though they are not a part of the TRE core. These results, which are consistent with previous results obtained by mobility-shift assays (35), demonstrate that sequences that flank the TRE are also important for recognition by Jun proteins.

DISCUSSION

Collectively, these results indicate that m-Jun specifically recognizes the TRE *in vitro* and *in vivo*. Although it binds DNA as a monomer, m-Jun interacts with its recognition sites indistinguishably from c-Jun. These results are striking, considering the fact that the second basic region of m-Jun is polymerized in the C-terminal to N-terminal direction. These findings underscore the inherent flexibility of the basic region as a DNA-binding motif. A variety of experiments suggest that before DNA binding the basic region is unstructured but assumes a helical structure after DNA binding (18, 19, 32, 33). In addition to the structural transition of the basic region upon interaction with its target, the target sequence itself undergoes bending, resulting in even a better fit between the DNA and protein (36). Our results indicate that the polypeptide backbone of the basic region is not involved in sequence recognition. The polypeptide backbone does not appear directly involved in contacting the DNA in most other DNA-binding proteins, the structure of which has been determined at high resolution (37). However, we demonstrate that a DNA sequence-recognition motif can be polymerized in a polarity opposite to that of the natural structure and still maintain its activity and specificity. Even though m-Jun still contains one normal basic region that probably makes an important contribution to binding, the footprinting and methylation interference experiments indicate that both halves of

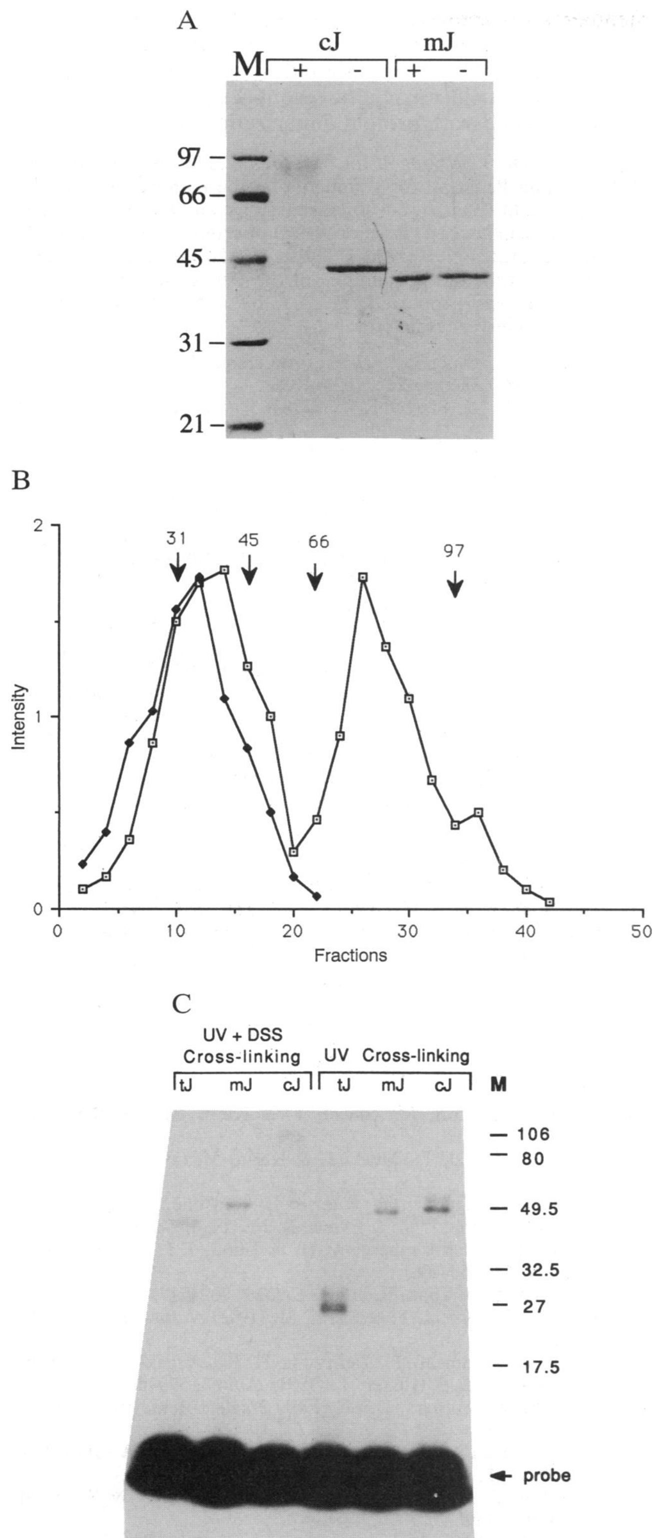

FIG. 3. m-Jun is a monomer in solution and upon binding to DNA. The aggregation state of m-Jun (mJ) was compared with that of c-Jun (cJ) by chemical cross-linking (*A*) and sedimentation (*B*) analyses. In *A* −, dimethyl sulfoxide; +, DSS/dimethyl sulfoxide. In *B* the graph shows relative concentrations of c-Jun (□) and m-Jun (◆) in different fractions, as determined by densitometry with an LKB UltroScan XL and peak positions of the molecular mass markers. (*C*) Aggregation state of protein–DNA complexes formed by m-Jun (mJ), t-Jun (tJ), and c-Jun (cJ) was analyzed by UV and DSS cross-linking. m-Jun (200 ng), t-Jun (40 ng), and c-Jun (40 ng) were incubated with ^{32}P-labeled *jun*–TRE probe (1 ng) for 20 min and then subjected to cross-linking by either UV alone or UV plus DSS. M, molecular mass markers.

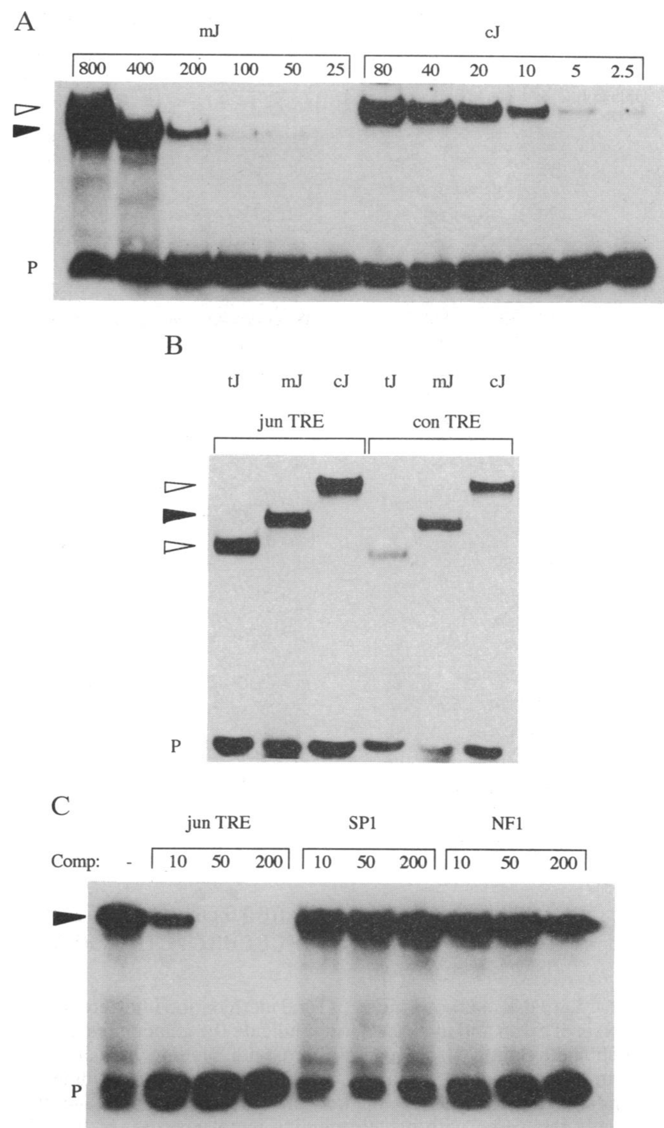

FIG. 4. Mobility-shift assays. (*A*) Protein titration experiment. A fixed amount (1 ng) of end-labeled *jun*–TRE probe (P) was incubated with increased amounts of c-Jun (cJ) and m-Jun (mJ), as indicated (in ng). Formation of protein–DNA complexes (solid arrow for m-Jun and open arrow for c-Jun) was analyzed by the mobility-shift assay. (*B*) Full-length c-Jun (cJ), truncated c-Jun (tJ), and m-Jun (mJ) were incubated with *jun*–TRE and consensus (con) TRE probes; the protein–DNA complexes (solid arrow for m-Jun, open arrows for c-Jun and t-Jun) were separated from free probes (P) by electrophoresis on a nondenaturing polyacrylamide gel. (*C*) Competition (Comp) experiment. m-Jun (400 ng) was incubated with 1 ng of the *jun*–TRE probe in the presence of the indicated amount (in ng) of unlabeled *jun*–TRE, Sp1, and NF1-binding-site oligonucleotides.

the TRE are contacted by the protein in a similar manner and to the same extent. Thus, it appears possible that as long as the basic region can project the same order of side chains into the major groove, it can bind DNA in a sequence-specific manner. These findings are encouraging for the future design of synthetic DNA-binding domains and suggest that such domains could be generated by anchoring appropriate side chains into a flexible polymeric backbone other than a polypeptide. The use of a nonpolypeptide backbone is likely to increase the biological half-life of the polymer, as it will not be recognized by cellular proteases.

Although transactivation by m-Jun was considerably lower than transactivation by c-Jun, immunoprecipitation indicates that m-Jun was also expressed less efficiently than c-Jun.

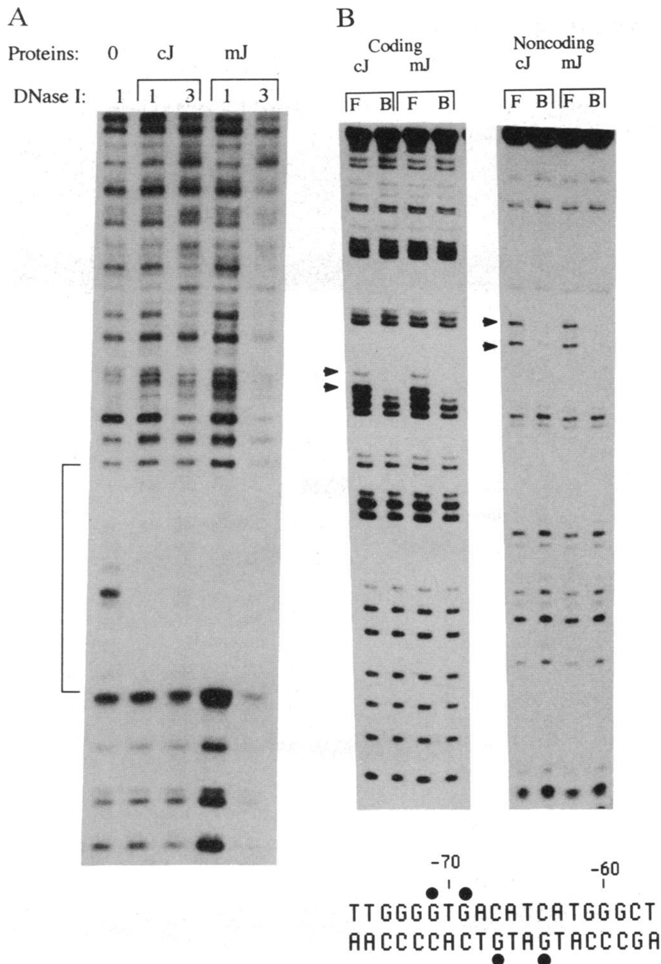

FIG. 5. DNase I footprinting (*A*) and methylation interference (*B*) analysis of c-Jun and m-Jun. Arrows indicate the guanine residues at which methylation strongly interfered with binding of c-Jun and m-Jun. Location of these guanines within the c-*jun* AP-1-binding site is indicated by circles at the bottom. The guanine residue that partially interfered with protein binding is not marked. F, free; B, bound.

Taking into consideration the 8-fold difference in the level of expression of the two proteins, m-Jun could function *in vivo* almost as efficiently as c-Jun. We noticed that another monomeric c-Jun construct with a loop only two amino acids shorter than m-Jun cannot transactivate the *jun* promoter (Fig. 1*C*). A small protein analogous to m-Jun was described by Talanian *et al.* (19), who connected two GCN4 (responsible for general control of amino acid biosynthesis in yeast) basic-region peptides via a disulfide bridge. Although this protein bound DNA at 4°C *in vitro*, it is unlikely that the disulfide bridge will remain oxidized at higher physiological temperatures and the reducing intracellular environment.

Our results strongly suggest that the only function of the leucine zipper is to mediate protein dimerization. As long as two basic regions can be tethered together at the right geometry, the leucine zipper is not required for either transactivation or for conferring binding specificity.

The approach described here can be used to assess the ability of other bZIP and probably also helix–loop–helix proteins to activate transcription by binding to their natural recognition sites. This is an important test because the ability of a given protein to activate transcription may depend on the binding-site type with which it interacts (5, 7, 8). Because these proteins will not be able to interact with other family members, this approach would reveal their intrinsic activity. Finally, the availability of monomeric derivatives of sequence-specific activators should simplify their structural analysis with nuclear magnetic resonance by alleviating problems associated with protein dimerization.

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