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Cooperative Interaction of GATA-2 and AP1 Regulates Transcription of the Endothelin-1 Gene

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Endothelin-1 (ET-1) is a 21-amino-acid vasoactive peptide initially characterized as a product of endothelial cells. Reporter gene transfection experiments have indicated that a GATA site and an AP1 site are essential for ET-1 promoter function in endothelial cells, and GATA-2 appears to be the active GATA factor which regulates ET-1 expression. To look for interactions between AP1 and GATA-2, transactivation experiments were performed with expression vectors encoding c-Jun, c-Fos, and GATA-2. Cooperativity between the AP1 complex and GATA-2 was observed as a synergistic increase in transcriptional activity of the ET-1 reporter plasmid. In addition, AP1 was able to potentiate the action of GATA-2 on reporter constructs lacking a functional AP1 site. In a similar fashion, GATA-2 was able to potentiate the action of AP1 despite deletion of the GATA site. Experiments with GATA-1 and GATA-3 expression vectors provided evidence that this capacity to interact with AP1 may be a characteristic of all GATA family members. Biochemical evidence for AP1-GATA interaction was provided by immunoprecipitation experiments. A GATA-2-specific antiserum was shown to immunoprecipitate in vitro-synthesized Jun and Fos protein from reticulocyte lysate. Also, antisera directed against Jun and Fos were able to immunoprecipitate from nuclear extracts a GATA-binding protein, indicating the association of AP1 and GATA proteins in vivo.

Endothelin-1 (ET-1) is a potent vasoconstrictor and smooth muscle cell mitogen expressed in endothelial cells of the vascular wall (38). Because of the potential role of ET-1 in vascular diseases such as hypertension and atherosclerosis, the biology of this vasoactive factor and its high-affinity receptor has been intensively studied (21, 22). In particular, numerous studies have investigated the regulation of the ET-1 gene by hormones and physical factors such as hypoxia and shear stress (10).

This laboratory and others have used the ET-1 gene to investigate transcriptional regulation in endothelial cells. The high-level cell-restricted expression of this promoter in vitro has made it an attractive model for such studies (4, 17–19, 36). Reporter gene transfection experiments have indicated that the ET-1 promoter has at least two essential protein binding motifs (17, 36). One of these (TTATCT, bp −136 to −131) is similar to sequences associated with other eukaryotic genes which interact with one or more GATA factors. Members of the family of GATA-binding proteins recognize a consensus sequence motif, WGATAR, through a conserved zinc finger DNA binding domain (25). The GATA-1 protein is essential for development of both primitive and definitive erythroid cell lineages (5, 27, 32), and GATA-3 is considered to play an important role in lymphoid cell development (11, 13, 16, 37). Recently, a fourth member of the GATA family, GATA-4, has been identified and shown to be expressed primarily in the endocardium and myocardium (1, 8, 14). Expression studies with *Xenopus* embryos and gene targeting experiments with mice have established that GATA-2 has a very early role in hematopoietic cell development (15, 31, 34). Data from this laboratory and others have also indicated that GATA-2 serves an important function in transcriptional regulation of endothelial cell genes such as the ET-1 gene (4, 19). However, its presence alone cannot account for cell-restricted expression of ET-1, since GATA-2 appears to be expressed in many cell types in the adult (4, 19, 25, 37).

A second essential protein binding motif (TGACTAA, bp −108 to −102) is capable of mediating the binding of Jun and Fos family members and confers AP1 responsiveness on a heterologous promoter (18). In addition, this element appears essential for constitutive baseline activity of the ET-1 promoter in endothelial cells in culture. Gel mobility shift assays have failed to provide evidence that factors other than Jun and Fos are capable of interacting with this site in endothelial cells (unpublished observations).

Much recent work has emphasized the importance of the interaction of transcription factors in the regulation of gene expression. Protein-protein interactions have recently been shown to provide a link between signal transduction pathways historically felt to terminate at independent transcription factors. Several members of the steroid receptor superfamily have been shown to interact with members of the Jun and Fos families (3, 6, 12, 24, 26, 28–30, 39). Myogenic determining factors have also been shown to interact with Jun and Fos family members, with this interaction modulating the balance between growth and differentiation (2, 20). Several lines of evidence indicate that GATA factors may interact directly with AP1 to regulate transcription. First, GATA and AP1 sites are found in close association in the core hypersensitivity sites of the globin locus control regions and in a number of erythroid promoters (23, 35). While some of these AP1-like sites may preferentially bind the erythroid factor NFE2, it is likely that some of these sites function primarily as Jun and Fos binding sites (25). Second, a number of experiments indicate that GATA sites in conjunction with an AP1 site constitute an

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active promoter, while GATA sites in conjunction with a TATA box alone do not form an active promoter (18, 35).

In this report, we have investigated the interaction between GATA-2 and Jun and Fos. Transfection analysis revealed a synergistic transactivation of the ET-1 promoter in endothelial cells when GATA-2 and Jun and Fos expression plasmids were combined. This synergism was observed even if the AP1 site of the ET-1 promoter was mutated, suggesting Jun and Fos may directly associate with GATA-2. Experiments using synthetic promoters confirmed these transfection data. Further transfection experiments revealed that other members of the GATA family, including GATA-1 and GATA-3, were able to cooperate with AP1 in the activation of the ET-1 promoter. Finally, we have used immunoprecipitation experiments to provide evidence that Jun and Fos proteins can associate with GATA proteins in vitro and in vivo.

MATERIALS AND METHODS

Cell culture. Bovine aortic endothelial cells (BAEC) were isolated and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (HyClone), 600 μg of glutamine per ml, 100 U of penicillin per ml, and 100 μg of streptomycin per ml as described previously (17). BAEC were passaged every 3 to 5 days, and cells at passages 6 to 8 were used for transfection experiments.

Plasmids. ET-1 reporter fusion plasmids contained the chloramphenicol acetyltransferase (CAT) gene in conjunction with ET-1 5′ promoter sequence. The reporter plasmid −204CAT contained 204 bp of the wild-type ET-1 promoter. Reporter plasmids with mutations in the GATA binding site (mutGATA), the AP1 site (mutAP1), or both (mutAP1mutGATA) were derived from −204CAT by PCR-based site-directed mutagenesis (9). In the mutGATA construct, the GATA binding sequence TTATCT was altered to TGCACT; in the mutAP1 reporter, the wild-type sequence GTGACTAA was altered to GG TACTAA. The mutAP1mutGATA construct combined these mutations. The sequence of each of the mutated PCR fragments was confirmed by dideoxy-chain termination sequencing. The synthetic CAT reporter construct (1A+3AP1) pSPCAT was constructed by cloning oligonucleotides encoding a single ET-1 GATA site and three tandem copies of the ET-1 AP1 site upstream of the simian virus 40 minimal promoter and has been previously described (19).

Expression plasmids encoding rat c-Fos (CMVFos) and rat c-Jun (CMVJun) were provided by Tom Curran (Roche Institute of Molecular Biology, Nutley, N.J.). GATA-1 and GATA-3 expression plasmids were the kind gifts of C. Trainer (National Institutes of Health, Bethesda, Md.) and D. Engel (Department of Biochemistry, Northwestern University, Evanston, Ill.), respectively. The GATA-2 expression plasmid (DT3A) was cloned in this laboratory and has been previously described (19). A partial-length clone encoding human E12 was kindly provided by C. Murre (Department of Biology, University of California, San Diego).

Transfection and CAT assays. BAEC in 100-mm-diameter dishes were transfected with DNAs which had been incubated with 50 μg of Lipofectin (Bethesda Research Laboratories). Each dish of cells received 5 μg of CAT construct and 1 to 2 μg of each expression plasmid. As a means for correcting for transfection efficiency, either a pSVβgal or pRSVβgal construct was also included in each transfection. The quantity of DNA in each transfection was increased to a total of 10 μg by the addition of pCDM8 vector plasmid DNA. Transfection was carried out by incubating cells in the presence of DNA for 4 h. Subsequently, cells were washed and cultured in fresh medium containing 2% fetal calf serum. Cell extracts were prepared 48 h after transfection. The CAT and β-galactosidase assays were performed as previously described (17). CAT activity was normalized to β-galactosidase activity, and transactivation was expressed as fold increase over the activity of the reporter construct alone (relative CAT activity). Transfection experiments were repeated at least 10 times. Since there was little variation between experiments, the data for a single representative experiment are presented as the mean of a triplicate transfection.

Gel mobility shift assays. Complementary oligonucleotides encoding the ET-1 GATA site were labeled with T4 polynucleotide kinase and used in binding reactions with 2 μl of commercially obtained rabbit reticulocyte lysate (Promega), and nondenaturing gel electrophoresis was performed as previously described (18).

In vitro protein synthesis and immunoprecipitations. Anti-Jun and anti-Fos polyclonal antisera were provided by S. Hann (Cell Biology, Vanderbilt University, Nashville, Tenn.). Polyclonal antisera directed against the carboxy-terminal 289 amino acids of human GATA-2 (α-GATA-2a) or the amino-terminal 154 amino acids (α-GATA-2b) were generated in this laboratory. GATA-2 cDNA sequence 5′ or 3′ of the internal *Sac*I site was adapted with a synthetic linker for insertion in frame into the *Eco*RI site of the prokaryotic expression vector pMal-c (New England Biolabs). Recombinant fusion protein was induced in the presence of isopropylthiogalactopyranoside (IPTG), and the induced fusion proteins containing maltose-binding protein and GATA-2 were excised from the gel. The gel fragments were homogenized into Freund's complete adjuvant and injected into rabbits as described elsewhere (7). Specificity of the antisera was evaluated by Western blotting (immunoblotting) using gels containing intact and cleaved maltose-binding protein–GATA-2 and unrelated fusion proteins.

^{35}S-labeled proteins were synthesized in vitro, using rabbit reticulocyte lysate (Promega) under conditions suggested by the manufacturer. Lysate reaction mixtures containing c-Jun, c-Fos, E12, and GATA-2 were incubated at 37°C for 30 min, and then 500 μl of radioimmunoprecipitation assay (RIPA) buffer and 5 μl of antisera were added. Following incubation for 2 h at 4°C, 30 μl of precleared protein A-Sepharose (Sigma) was added, and this mixture was incubated for an additional 2 h at the same temperature. The samples were washed extensively in RIPA buffer and 2 M urea. Bound proteins were eluted in sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels, and the gels were fixed, dried, and exposed to film.

Protein-DNA cross-linking and immunoprecipitation. BAEC were stimulated in Dulbecco's modified Eagle's medium plus 20% fetal calf serum for 2 h, washed three times with phosphate-buffered saline (PBS), scraped, and collected in PBS at 4°C by centrifugation. Cells were suspended in an equal volume of extraction buffer (20 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [HEPES; pH 7.9], 600 mM KCl, 20% glycerol, 1 mM EDTA, 100 μg of aprotinin per ml) and lysed by freezing and thawing three times. Lysed cells were centrifuged, and the supernatants aliquoted, quickly frozen, and stored at −80°C. The DNA binding reaction was conducted in a total volume of 200 μl with 50 μl of cell extract and 500,000 cpm of ^{32}P-labeled complementary oligonucleotides encoding the ET-1 GATA site (GCCTTATCTCCGGCTGC) or an SP1 binding site (ATTCGATCGGGGCGGGGCGAG). Also included in the binding reaction was 10 μg of poly(dI-dC) and a standard binding buffer (18). Each reaction mixture was irradiated on ice with a UV source (254 nm, 80 kJ/m^2). One milliliter of RIPA buffer was then added to each sample along with the appropriate polyclonal antiserum (α-GATA-2b, anti-Jun, anti-Fos, or a control antiserum). These reactions mixtures were incubated for 1 h at 4°C, protein A-Sepharose was added, and incubation continued for an additional 2 h. Finally, the samples were washed in RIPA buffer, boiled, and subjected to SDS-PAGE (10% polyacrylamide gels), and the dried gels were subjected to autoradiography.

RESULTS

GATA-2 and AP1 interact, and this interaction is not dependent on the presence of both protein binding motifs. Previous studies have shown that overexpression of GATA-2 increases transcription of ET-1 promoter constructs containing the GATA site and that overexpression of c-Jun and c-Fos increases transcription of ET-1 promoter constructs containing the AP1 site (4, 18, 19). Results of experiments reported here, using a 204-bp ET-1 promoter construct, confirm these earlier findings (Fig. 1A). Transfection of a GATA-2 expression plasmid resulted in a 3-fold increase in transcription (Fig. 1A, bars 1 and 2), and transfection of Jun and Fos expression plasmids resulted in an 18-fold increase in transcription (bars 1 and 3). A new finding was that simultaneous cotransfection of expression constructs encoding all three transcription factors led to a greater than additive (80-fold) increase in transcription (bars 1 and 4).

This apparent cooperativity of AP1 and GATA transcription factors was further investigated by using the 204-bp ET-1 promoter with mutations in the AP1 site (mutAP1; Fig. 1B). As previously described, this 2-bp mutation in the AP1 site decreases protein binding to this region of the promoter and leads to a decrease in basal promoter activity (18). The decrease in baseline promoter activity is also seen in these experiments (compare lanes 1 in Fig. 1A and B; note the difference in ordinate scales). Cotransfection of the GATA-2 expression plasmid resulted in an increase in transcription similar to that seen with the wild-type promoter (Fig. 1B, bar 2). Despite the absence of a functional AP1 site, this reporter construct also responded with a significant increase in transcription to the overexpression of Jun and Fos (bars 1 and 3). The ability of Jun and Fos expression vectors to augment the effect of GATA-2 was also maintained in this mutAP1 reporter construct (bars 1 and 4).

The increase in transcription of the mutAP1 promoter by

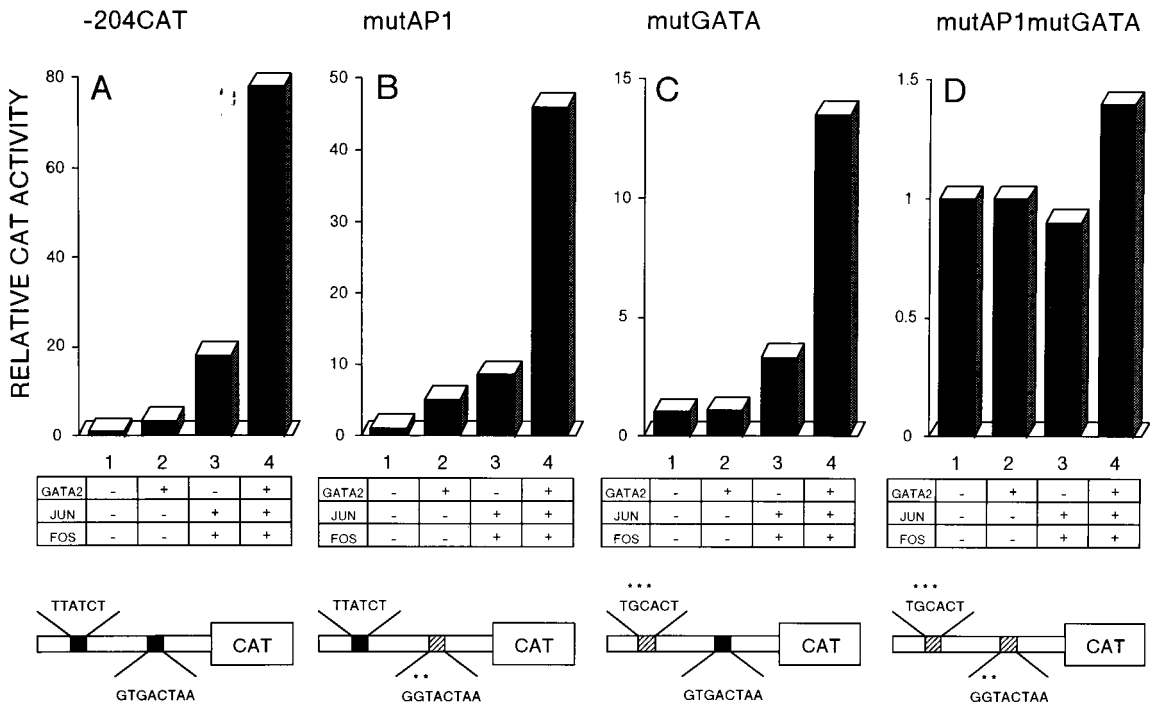

FIG. 1. Synergistic transactivation of the ET-1 promoter by GATA-2 and Jun and Fos. BAEC were transfected with expression plasmids encoding human GATA-2, rat c-Jun, and rat c-Fos. The reporter constructs contained the CAT gene under control of the ET-1 promoter with or without mutations of the GATA and AP1 DNA binding sequences. (A) A native 204-bp ET-1 promoter linked to the CAT gene (−204CAT) served as the reporter gene. Five micrograms of reporter DNA was transfected with 1 to 2 μg of DNA for each expression vector. A constitutive β-galactosidase vector was included in each transfection to control for differences in efficiency of transfection. CAT activity was normalized to β-galactosidase activity, and transactivation was expressed as fold increase over the activity of the reporter construct alone. (B) Reporter plasmid mutAP1 used in these experiments differed from −204CAT by 2 mutated bp in the AP1 site. Transfection conditions were the same as for panel A. (C) The reporter gene mutGATA differed from −204CAT by 3 mutated bp in the GATA site. Transfection conditions were the same as for panel A. (D) The reporter plasmid mutAP1mutGATA combined mutations found in mutGATA and mutAP1 constructs. Transfection conditions were the same as for panel A.

Jun and Fos was felt likely to be mediated by the GATA site or by cryptic AP1 sites remaining in the ET-1 promoter or the parent plasmid. To distinguish between these possibilities, we conducted experiments with a 204-bp ET-1 promoter with mutations in both the AP1 site and the GATA site (mutAP1mut GATA; Fig. 1D). First, it is interesting that deletion of these two sites results in a dramatic decrease in basal promoter activity (Fig. 1D, bar 1; note the difference in ordinate scale). Cotransfection of Jun and Fos expression plasmids did not result in a measurable increase in promoter activity, providing evidence against residual cryptic AP1 sites (bar 3). These data indicate that the ability of Jun and Fos to increase transcription of the mutAP1 construct, and the ability to enhance the transactivation of GATA-2, depends on an intact GATA site. Since Jun and Fos cannot directly bind to the GATA site (unpublished observations), it seems most likely that these factors influence the rate of transcription via protein-protein interaction. In this context, Jun and Fos may interact with endogenous GATA-2 and increase transcription of mutAP1 through the GATA binding site (Fig. 1B, bar 2).

To further investigate AP1–GATA-2 cooperativity, transactivation experiments were performed with a 204-bp ET-1 promoter containing a mutated GATA site (mutGATA; Fig. 1C). Cotransfection of the GATA-2 expression vector failed to increase the level of transcription of this reporter construct (Fig. 1C, bars 1 and 2). This result indicates that GATA-2 alone is no longer able to activate this promoter. Jun and Fos were able to transactive this mutated promoter at the expected level (bar 3). However, when the GATA-2 expression plasmid was cotransfected with those encoding Jun and Fos, we detected a moderate but consistent increase in activation which was greater than that observed with Jun and Fos alone (bars 3 and 4). This increase is seen in the presence of the GATA-2 expression vector, despite baseline levels of endogenous GATA-2 in these BAEC in culture. Since GATA-2 alone did not lead to an increase in transcription, it seems likely that it functions to amplify the response of Jun and Fos which is mediated via the remaining AP1 site. Since endothelial cells have very low baseline levels of AP1 binding activity (data not shown), it is not expected that GATA-2 alone would be functional via such an interaction with endogenous Jun and Fos at the AP1 site.

The apparent ability of GATA-2 and AP1 to increase transcription of the reporter construct despite mutation of their cognate binding sites could potentially result from cross talk between the expression constructs. For instance, results with the mutAP1 and mutGATA reporter constructs (Fig. 1B and C) could reflect the action of transcription factors encoded by one expression vector on the promoters of the other expression vectors. GATA-2 might increase expression of the cytomegalovirus promoters which drive the Fos and Jun expression plasmids. This possibility was made less likely by using saturating amounts of each expression construct. In titration experiments, increasing amounts of the expression vectors were transfected in conjunction with the ET-1 reporter plasmid. With both the GATA-2 and the Jun and Fos expression plasmids, a plateau was reached after which increased amounts of the expression plasmid did not result in increased levels of transcription (data not shown). These plateau levels were 2 μg for the GATA-2 construct and 1 μg for the Jun and Fos

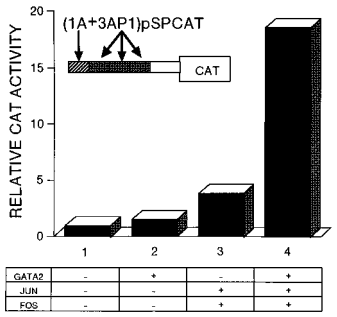

FIG. 2. Synergistic transactivation of a synthetic promoter by GATA-2 and Jun and Fos. The CAT reporter construct for these experiments, (1A+3AP1) pSPCAT, contained oligonucleotides encoding a single copy of the ET-1 GATA binding sequence and three tandemly arranged copies of the ET-1 AP1 site cloned upstream of the minimal simian virus 40 promoter in plasmid pSPCAT. GATA-2, Jun, and Fos expression plasmids and transfection methods were as described for Fig. 1.

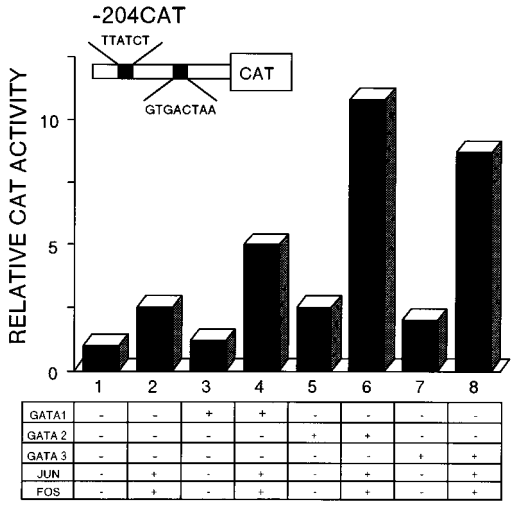

FIG. 3. Interaction between Jun and Fos and other GATA protein family members. BAEC were transfected with the native ET-1 promoter construct −204CAT. Human GATA-1, GATA-2, and GATA-3 expression vectors were transfected with or without Jun and Fos expression vectors.

constructs. These amounts of plasmid were used in the transactivation experiments to help ensure that transcription factors expressed from one construct did not influence expression of the other constructs.

Additional transfection experiments investigated the interaction of GATA-2 and AP1 in the context of a synthetic promoter reporter construct. These experiments were performed with a construct containing the minimal simian virus 40 promoter combined with oligonucleotides encoding a single GATA site and three copies of the ET-1 AP1 sequence (19). Cotransfection of the GATA-2 expression vector with this reporter resulted in a small but consistent increase in transcription, similar to that seen with the native ET-1 promoter (Fig. 2). The Jun and Fos expression vectors increased the rate of reporter transcription by approximately fourfold. Cotransfection of the GATA-2 and the Jun and Fos expression vectors shows a consistent 20-fold increase over the baseline level, a greater activation than expected from the additive effects of these factors. While this experiment does not address the mechanism of the cooperativity, it provides further support for the cooperative interaction of these transcription factors.

Interaction between Jun and Fos and other GATA protein family members. Transcription factors which bind to the GATA motif constitute a family of homologous zinc finger proteins (1, 14, 25). There is a high degree of amino acid homology in the zinc finger DNA binding domains of these proteins, and this correlates with the high degree of conservation of the cognate DNA binding sequences. However, amino acid sequence outside of the zinc fingers is less highly conserved and may reflect differences in the profiles of protein-protein interactions that allow these proteins to modulate transcription in different cell types. To investigate the potential for GATA-1 and GATA-3 to interact with Jun and Fos, expression vectors encoding these factors were cotransfected with those encoding Jun and Fos. The wild-type −204CAT reporter construct was used in these experiments with endothelial cells. In these experiments, GATA-1 and GATA-3 expression plasmids also showed the synergistic enhancement of ET-1 reporter gene expression when cotransfected with Jun and Fos expression plasmids (Fig. 3, bars 4 and 8). While GATA-1 was only minimally active toward this nonerythroid promoter in endothelial cells, the enhancement of transcription in the presence of Jun and Fos is easily seen (bars 3 and 4). GATA-3 alone is intermediate between GATA-1 and GATA-2 in its ability to activate the ET-1 promoter construct when transfected alone but is equal to GATA-2 in its ability to potentiate the effect of Jun and Fos (bars 7 and 8). These data indicate that the interaction of Jun and Fos with GATA-binding proteins is not limited to GATA-2, suggesting that this interaction might be a general mechanism of transcriptional regulation involving all of the GATA factors.

Physical association of Jun and Fos and GATA proteins. Results of transfection experiments indicating that Jun and Fos and GATA-2 are able to increase transcription despite deletion of their cognate binding sites suggest that the cooperativity seen between these factors may be mediated by direct protein-protein interaction. To investigate this possibility, experiments were performed to provide biochemical evidence for the association of these proteins in solution. These experiments were made possible by the development of polyclonal rabbit antisera raised against portions of recombinant GATA-2 protein. The authenticity of these antisera was demonstrated by their ability to recognize recombinant GATA-2 protein on Western blots and ability to immunoprecipitate a 49-kDa protein from metabolically labeled endothelial cells (data not shown).

To perform in vitro biochemical studies investigating the interaction of GATA-2 with Jun and Fos, these proteins and an unrelated control protein (E12) were individually synthesized in reticulocyte lysate reactions using mRNA derived from cDNA clones (Fig. 4A). Radiolabeled Jun and Fos proteins were immunoprecipitated by specific antisera but not by preimmune serum (Fig. 4B, lanes 1, 2, 4, and 5). However, the GATA-2 antibody was ineffective at precipitating the synthetic GATA protein (Fig. 4B, lane 8). It seemed likely that the GATA antiserum was neutralized by rabbit GATA factors endogenous to the reticulocyte lysate, and specific experiments were conducted to verify this possibility.

Gel mobility shift assays were conducted with complementary oligonucleotides encoding the ET-1 GATA site (Fig. 4C). As observed with similar experiments using this DNA probe with nuclear extracts, two retarded complexes are visualized. The larger of these has been shown to represent specific GATA binding activity (data not shown). This GATA binding activity in reticulocyte lysate is likely due in part to GATA-1, but the presence of GATA-2 and GATA-3 in immature erythrocytes raises the possibility that these proteins are present as

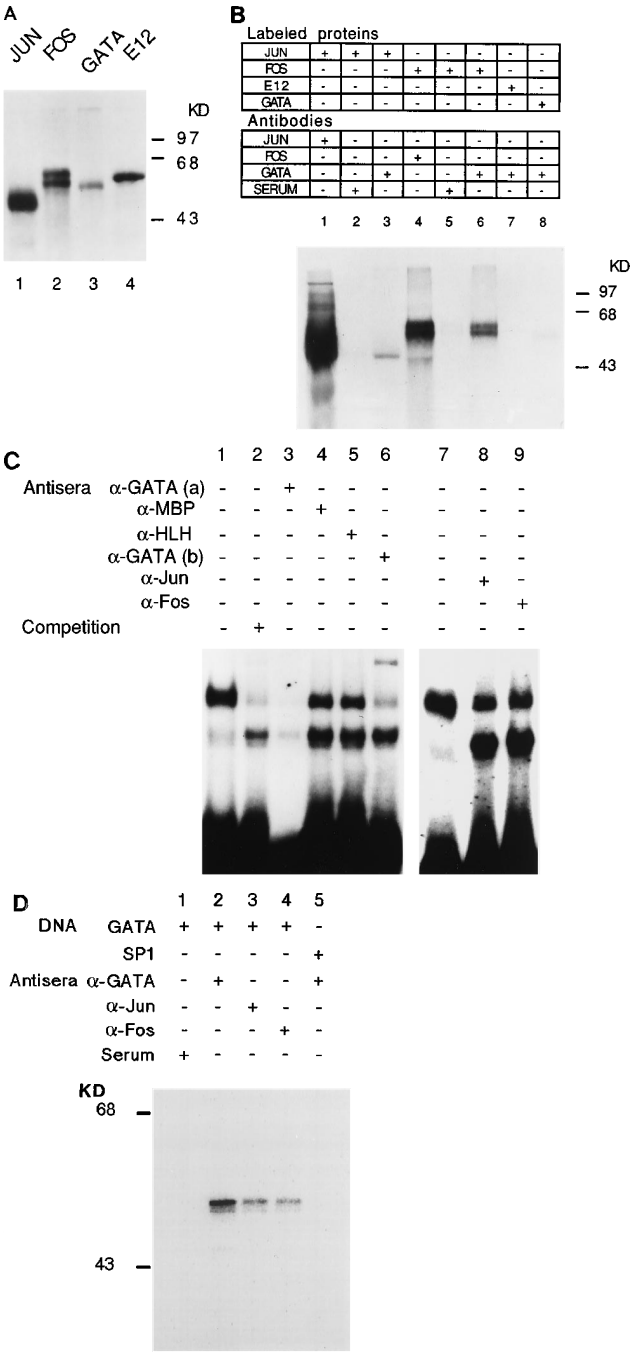

FIG. 4. Specific in vitro and in vivo association of Jun, Fos, and GATA proteins. (A) [^{35}S]methionine-labeled c-Jun, c-Fos, E12, and GATA-2 proteins were produced by translation in rabbit reticulocyte lysate of in vitro-transcribed mRNAs. In vitro translated products were analyzed as the input of immunoprecipitation experiments shown in panel B by SDS-PAGE (10% polyacrylamide gel). Autoradiography is shown. (B) ^{35}S-labeled proteins were incubated with c-Jun, c-Fos, or GATA antibodies for 2 h at 4°C. Protein A-Sepharose was added, and the mixture was incubated for another 2 h at 4°C. The samples were washed with RIPA buffer and urea, eluted with SDS sample buffer, and subjected to SDS-PAGE (10% polyacrylamide gel). The gel was fixed and dried; autoradiography is shown. Although the same amounts of counts were used in all experiments, the number of counts loaded in the Jun immunoprecipitation was greater, thus accounting for the prominent band in the gel shown (lane 1). (C) Verification that GATA-2 antisera recognize rabbit GATA factors endogenous to the reticulocyte lysate. Gel mobility shift assays were performed with reticulocyte lysate, using radiolabeled complementary oligonucleotides encoding the ET-1 GATA binding site as a probe. Two retarded complexes were formed, and the upper complex was shown to represent specific binding by competition with excess unlabeled oligonucleotides. When α-GATA-1a [α-GATA (a)] was included in the binding reaction, the specific binding was almost completely destroyed. α-GATA-1b [α-GATA (b)] did not interfere with protein-DNA association but increased the mass of the complex, producing a shift in the mobility pattern. Polyclonal antisera against Jun and Fos (α-Jun and α-Fos) did not alter specific binding to the ET-1 GATA sequence. Polyclonal antisera directed against maltose-binding protein (α-MBP) and a helix-loop-helix transcription factor (α-HLH) also failed to alter specific binding to the ET-1 GATA site. Lanes 1 to 6 and 7 to 9 represent two separate gel mobility shift experiments run under identical conditions. (D) In vivo association of GATA-2 and Jun and Fos proteins. Nuclear extracts from serum-stimulated BAEC were used in a binding reaction with oligonucleotides encoding the ET-1 GATA site or a consensus SP1 site. Protein-DNA complexes were UV cross-linked, and this reaction was subjected to immunoprecipitation with an antiserum specific for GATA-2, Jun, or Fos or a negative control antiserum. A major 52-kDa band and several smaller bands are seen with the GATA-2, Jun, and Fos antisera.

well (37). The GATA binding activity was almost abolished when an antiserum directed against the carboxy-terminal two-thirds of GATA-2 (α-GATA-2a) was included in the binding reaction, as evidenced by the disappearance of the upper retarded complex. An antiserum directed against the amino-terminal one-third of GATA-2 (α-GATA-2b) also recognized the GATA-binding protein, as evidenced by a supershifted complex. These results indicate that the GATA-2 antisera are capable of recognizing the GATA factors in reticulocyte lysate which bind the ET-1 GATA site. Given the high degree of conservation of amino acid sequence between species and between the DNA binding regions of the GATA factors, it is not surprising that the GATA-2 antisera were able to inactivate endogenous GATA-binding activity.

Binding experiments were also conducted in the presence of Jun and Fos antisera (Fig. 4C). As with negative control antisera (directed against maltose-binding protein and the helix-loop-helix transcription factor), there was an increase in nonspecific binding but no significant change in the GATA binding activity. These data verify the specificity of the antisera. Also, since these antisera are known to supershift DNA-binding complexes containing Jun and Fos proteins, these experiments provide evidence that Jun and Fos are not present in the DNA-protein complex or that the epitopes recognized by these antisera are masked by the interaction with GATA-binding proteins.

An experiment was thus conducted to investigate the ability of the ^{35}S-labeled Jun and Fos proteins to interact with GATA factors endogenous to the reticulocyte lysate. Immunoprecipitations were performed with the polyclonal antisera to GATA-2 and the in vitro-translated Jun and Fos proteins. Interestingly, a significant portion of the synthetic Fos protein was immunoprecipitated by the GATA antibodies (Fig. 4B, lane 6). Similarly, labeled Jun protein was also immunoprecipitated with the GATA antisera (lane 3). These findings do not result from the GATA-2 antisera directly interacting with Jun and Fos, since results of control experiments using recombinant Jun and cell extracts containing high levels of native Jun and Fos have verified the lack of such cross-reactivity (data not shown). Also, the unrelated helix-loop-helix transcription factor E12 was not immunoprecipitated by the GATA antisera (lane 7). These results indicate that in vitro-synthesized Jun and Fos proteins were capable of associating with endogenous GATA factors with such affinity that they were coprecipitated despite harsh washing conditions such as with 2 M urea. These GATA factors probably include GATA-1 and GATA-3 as well as GATA-2.

In view of the results of the transfection experiments presented above, such in vitro findings are suggestive of direct protein-protein interaction. To further explore this possibility,

in vivo experiments were conducted with protein harvested from cultured cells. Techniques used in these experiments were similar to those used to confirm the in vivo association of AP1 and the glucocorticoid receptor (12). A binding reaction was conducted with ^{32}P-labeled oligonucleotides encoding the ET-1 GATA site and endothelial cell nuclear protein with a serum-induced increase in AP1 binding activity. Molecules binding to the GATA site were UV cross-linked to the radiolabeled DNA and analyzed by immunoprecipitation and SDS-PAGE. When immunoprecipitation was performed with antisera to GATA-2 and protein cross-linked to the GATA site DNA, a band of the expected size (52 kDa) was observed on the autoradiograph (Fig. 4D, lane 2). The presence of multiple bands most likely represents posttranslational modification of the GATA-2 protein, although degradation during the experimental protocol also remains a possibility. Most importantly, a similar-size band was also observed when Jun and Fos antisera were used in the immunoprecipitation. This result indicates that Jun and Fos proteins are capable of associating with the GATA-2 protein which is cross-linked to GATA binding sequence. The specificity of this interaction is verified by control experiments using the SP1 binding site as a DNA probe (lane 5) and a control immune serum (lane 1).

DISCUSSION

Results of transactivation experiments presented here provide evidence that the combination of transcription factors GATA-2 and AP1 increases transcription in a greater than additive manner. This synergism was shown to occur with the native proximal ET-1 promoter and also for synthetic promoters combining GATA and AP1 cognate binding sites with a heterologous promoter in an independent plasmid backbone (Fig. 1 and 2). Other members of the GATA family of transcription factors, GATA-1 and GATA-3, were also shown to be able to cooperate with the AP1 complex. All transactivation experiments were conducted with saturating amounts of expression constructs, to rule out the possibility that the observed effects were due to cross-stimulation between the expression vectors. Taken together, these experiments provide strong evidence for the cooperative interaction of these transcription factors. While the combination of an AP1 site and a GATA site has been shown to provide a strong promoter activity (35), this type of cooperative interaction has not been previously described for AP1 and GATA factors.

In many cases, this type of synergistic activation of transcription has been attributed to improved binding characteristics of the involved proteins (33). Results of experiments using the mutated binding sites in the ET-1 promoter argue against simple cooperativity in binding to the cognate binding sites (Fig. 1). Both GATA-2 and AP1 expression constructs were able to increase transcription of the ET-1 promoter despite deletion of their binding sites. Thus, the cooperativity between GATA-2 and AP1 must reflect improved interaction with the basal transcription apparatus. One possibility is that cooperativity is due to the two proteins associating and exerting a combined effect which is much greater than the effect of either protein alone. Together, they may be more efficient at recruiting elements of the basal transcription apparatus or necessary cell-restricted factors. In this case, a GATA-2–AP1 protein complex would be able to interact with both GATA and AP1 binding sites. Alternatively, individual binding of GATA-2 or AP1 to the ET-1 promoter might establish an interaction with members of the basal transcription apparatus, subsequently allowing the free other factor to then be recruited into the active transcription complex. The two factors would then function in an independent but synergistic fashion through the transcription apparatus.

To distinguish between these possible mechanisms, we have conducted experiments to investigate the potential for direct association of these transcription factors. Immunoprecipitation experiments using GATA factors found in reticulocyte lysate and proteins translated in reticulocyte lysate have indicated that both Jun and Fos can associate with GATA factors in vitro. More compelling are the data for assays with nuclear extract, which indicate that GATA-2 protein cross-linked to the ET-1 GATA binding site can be immunoprecipitated by antisera directed against Jun and Fos. Such data provide evidence for the physical association of members of these protein families in endothelial cells and support the theory that cooperativity of action is mediated through the interaction of a GATA-2–AP1 protein complex with the transcription apparatus.

In view of the widespread expression of members of the GATA protein family and members of the Jun and Fos families, the potential for interaction of these protein families at the molecular level has significant implications. Modulation of the activity of Jun and Fos in response to a variety of extracellular signals has been shown to regulate the expression of a great number of eukaryotic genes (2, 3, 6, 12, 20, 24, 26, 28–30, 39). Any impact on these pathways by additional factors would provide for more complex regulation of transcription. Our understanding of the specific roles of GATA-AP1 interaction is hindered by the lack of information about the biological functions of GATA-2. GATA-1 and GATA-3 have restricted patterns of expression and have been implicated in hematopoietic and T-cell lineage determination, respectively (5, 11, 13, 16, 27, 32, 37). GATA-2, which is expressed in a wide variety of cell types and is found very early in development, remains poorly understood. While gene targeting experiments have established a role for GATA-2 in early hematopoietic cell development, this transcription factor likely has broader roles in transcriptional regulation in additional cell types (31).

What role does this interaction between GATA-2 and Jun and Fos play in the cell-restricted expression of the ET-1 gene? Transfection experiments have shown that a portion of the ET-1 promoter containing the GATA site and the AP1 site is capable of conferring on a heterologous promoter enhanced expression in endothelial cells (17). Since GATA-2 and the Jun and Fos family members are widely expressed, the action of these factors alone cannot be responsible for such cell-restricted behavior. However, the binding of factors to both of these sites may allow the recruitment of additional proteins. While such proteins may be particularly important for the cell-restricted expression of the ET-1 promoter, they may not directly bind DNA and thus would not be directly detected with standard techniques commonly used in the study of DNA-binding transcription factors. Experiments to search for such accessory transcription factors in endothelial cells may provide a profitable avenue for further research.

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REFERENCES

1. **Arceci, R. J., A. A. J. King, M. C. Simon, S. H. Orkin, and D. B. Wilson.** 1993. Mouse GATA-4: a retinoic acid-inducible GATA-binding transcription factor expressed in endodermally derived tissues and heart. Mol. Cell. Biol. **13:**2235–2246.
2. **Bengal, E., L. Ransone, R. Scharfmann, V. J. Dwarki, S. J. Tapscott, H. Weintraub, and I. M. Verma.** 1992. Functional antagonism between c-Jun and MyoD proteins: a direct physical association. Cell **68:**507–519.
3. **Diamond, M. I., J. N. Miner, S. K. Yoshinaga, and K. R. Yamamoto.** 1990. Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Science **249:**1266–1272.
4. **Dorfman, D. M., D. B. Wilson, G. A. Bruns, and S. H. Orkin.** 1992. Human transcription factor GATA-2. Evidence for regulation of preproendothelin-1 gene expression in endothelial cells. J. Biol. Chem. **267:**1279–1285.
5. **Evans, T., and G. Felsenfeld.** 1989. The erythroid-specific transcription factor Eryf1: a new finger protein. Cell **58:**877–885.
6. **Gaub, M. P., M. Bellard, I. Scheuer, P. Chambon, and P. Sassone-Corsi.** 1990. Activation of the ovalbumin gene by the estrogen receptor involves the Fos-Jun complex. Cell **63:**1267–1276.
7. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
8. **Heikinheimo, M., J. M. Scandrett, and D. B. Wilson.** 1994. Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. Mol. Cell. Biol. **164:**361–373.
9. **Higuchi, R., B. Krummel, and R. K. Saiki.** 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16:**7351–7367.
10. **Hilkert, R., M.-E. Lee, and T. Quertermous.** 1992. Genetic regulation of endothelin-1 in vascular endothelial cells. Trends Cardiovasc. Med. **2:**129–133.
11. **Ho, I. C., P. Vorhees, N. Marin, B. K. Oakley, S. F. Tsai, S. H. Orkin, and J. M. Leiden.** 1991. Human GATA-3: a lineage-restricted transcription factor that regulates the expression of the T cell receptor alpha gene. EMBO J. **10:**1187–1192.
12. **Jonat, C., H. J. Rahmsdof, K.-K. Park, A. C. B. Cato, S. Gebel, H. Ponta, and P. Herrlich.** 1990. Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell **62:**1189–1204.
13. **Joulin, V., D. Bories, J. F. Eleouet, M. C. Labastie, S. Chretien, M. G. Mattei, and P. H. Romeo.** 1991. A T-cell specific TCR delta DNA binding protein is a member of the human GATA family. EMBO J. **10:**1809–1816.
14. **Kelley, C., H. Blumberg, L. I. Zon, and T. Evans.** 1993. GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. Development **118:**817–827.
15. **Kelley, C., K. Yee, R. Harland, and L. I. Zon.** 1994. Ventral expression of GATA-1 and GATA-2 in the *Xenopus* embryo defines induction of hematopoietic mesoderm. Dev. Biol. **165:**193–205.
16. **Ko, L. J., M. Yamamoto, M. W. Leonard, K. M. George, P. Ting, and J. D. Engel.** 1991. Murine and human T-lymphocyte GATA-3 factors mediate transcription through a *cis*-regulatory element within the human T-cell receptor delta gene enhancer. Mol. Cell. Biol. **11:**2778–2784.
17. **Lee, M. E., K. D. Bloch, J. A. Clifford, and T. Quertermous.** 1990. Functional analysis of the endothelin-1 gene promoter. Evidence for an endothelial cell-specific cis-acting sequence. J. Biol. Chem. **265:**10446–10450.
18. **Lee, M. E., M. S. Dhadly, D. H. Temizer, J. A. Clifford, M. Yoshizumi, and T. Quertermous.** 1991. Regulation of endothelin-1 gene expression by Fos and Jun. J. Biol. Chem. **266:**19034–19039.
19. **Lee, M. E., D. H. Temizer, J. A. Clifford, and T. Quertermous.** 1991. Cloning of the GATA-binding protein that regulates endothelin-1 gene expression in endothelial cells. J. Biol. Chem. **266:**16188–16192.
20. **Li, L., J. C. Chambard, M. Karin, and E. N. Olson.** 1992. Fos and Jun repress transcriptional activation by myogenin and MyoD: the amino terminus of Jun can mediate repression. Genes Dev. **6:**676–689.
21. **Masaki, T.** 1993. Homeostatic and compensatory actions of endothelins in circulating and endocrine systems. Endocrine Rev. **14:**256–268.
22. **Masaki, T., S. Kimura, M. Yanagisawa, and K. Goto.** 1991. Molecular and cellular mechanism of endothelin regulation. Implications for vascular function. Circulation **84:**1456–1468.
23. **Ney, P. A., B. P. Sorrentino, K. T. McDonagh, and A. W. Nienhuis.** 1990. Tandem AP-1-binding sites within the human beta-globin dominant control region function as an inducible enhancer in erythroid cells. Genes Dev. **4:**993–1006.
24. **Nicholson, R. C., S. Mader, M. Leid, C. Rochette-Egly, and P. Chambon.** 1990. Negative regulation of the rat stromelysin gene promoter by retinoic acid is mediated by an AP-1 binding site. EMBO J. **9:**4443–4454.
25. **Orkin, S. H.** 1992. GATA-binding transcription factors in hematopoietic cells. Blood **80:**575–581.
26. **Owen, T. A., R. Bortell, S. A. Yocum, S. L. Smock, M. Zhang, C. Abate, V. Shalhoub, N. Aronin, K. L. Wright, A. J. Wijnen, J. L. Stein, T. Curran, J. B. Lian, and G. S. Stein.** 1990. Coordinate occupancy of AP-1 sites in the vitamin D-responsive and CCAAT box elements by Fos-Jun in the osteocalcin gene: a model for phenotype suppression of transcription. Proc. Natl. Acad. Sci. USA **87:**9990–9994.
27. **Pevny, L., M. C. Simon, E. Robertson, W. H. Klein, S. F. Tsai, V. D'Agati, S. H. Orkin, and F. Constantini.** 1991. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature (London) **349:**257–260.
28. **Schule, R., P. Rangarajan, S. Kliewer, L. J. Ransone, J. Bolado, N. Yang, I. M. Verma, and R. M. Evans.** 1990. Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. Cell **62:**1217–1226.
29. **Schule, R., P. Rangarajan, N. Yang, S. Kliewer, L. J. Ransone, J. Bolado, I. M. Verma, and R. M. Evans.** 1991. Retinoic acid is a negative regulator of AP-1 responsive genes. Proc. Natl. Acad. Sci. USA **88:**6092–6096.
30. **Schule, R., K. Umesono, D. J. Mangelsdorf, J. Bolado, J. W. Pike, and R. M. Evans.** 1990. Jun-Fos and receptors for vitamins A and D recognize a common response element in the human osteocalcin gene. Cell **61:**497–504.
31. **Tsai, F.-Y., G. Keller, F. C. Kuo, M. Weiss, J. Chen, M. Rosenblatt, F. W. Alt, and S. H. Orkin.** 1994. An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature (London) **371:**221–226.
32. **Tsai, S. F., D. I. Martin, L. I. Zon, A. D. D'Andrea, G. G. Wong, and S. H. Orkin.** 1989. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature (London) **339:**446–451.
33. **Voss, J. W., L. Wilson, and M. G. Rosenfeld.** 1991. POU-domain proteins Pit-1 and Oct-1 interact to form a heteromeric complex and can cooperate to induce expression of the prolactin promoter. Genes Dev. **5:**1309–1320.
34. **Walmsley, M. E., M. J. Guille, D. Bertwistle, J. C. Smith, J. A. Pizzey, and R. K. Patient.** 1994. Negative control of *Xenopus* GATA-2 by activin and noggin with eventual expression in precursors of the ventral blood islands. Development **120:**2519–2529.
35. **Walters, M., and D. I. Martin.** 1992. Functional erythroid promoters created by interaction of the transcription factor GATA-1 with CACCC and AP-1/NFE-2 elements. Proc. Natl. Acad. Sci. USA **89:**10444–10448.
36. **Wilson, D. B., D. M. Dorfman, and S. H. Orkin.** 1990. A nonerythroid GATA-binding protein is required for function of the human preproendothelin-1 promoter in endothelial cells. Mol. Cell. Biol. **10:**4854–4862.
37. **Yamamoto, M., L. J. Ko, M. W. Leonard, H. Beug, S. H. Orkin, and J. D. Engel.** 1990. Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. Genes Dev. **4:**1650–1662.
38. **Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, and T. Masaki.** 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature (London) **332:**411–415.
39. **Yang-Yen, H.-F., J.-C. Chambard, Y.-L. Sun, T. Smeal, T. J. Schmidt, J. Drouin, and M. Karin.** 1990. Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction. Cell **62:**1205–1215.