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The Early Growth Response Protein (EGR-1) Regulates Interleukin-2 Transcription by Synergistic Interaction with the Nuclear Factor of Activated T Cells*

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The early growth response-1 gene (*EGR-1*) is induced by a wide range of stimuli in diverse cell types; however, EGR-1-regulated genes display a highly restricted pattern of expression. Recently, an overlapping Sp1·EGR-1 binding site has been identified within the interleukin-2 (IL-2) gene promoter directly upstream of the binding site for the nuclear factor of activated T cells (NFAT). We used transfection assays to study how the abundantly and constitutively expressed Sp1 protein and the immediate early EGR-1 zinc finger protein regulate *IL-2* gene expression. Here, we identify EGR-1 as an important activator of the *IL-2* gene. In Jurkat T cells, EGR-1 but not Sp1 acts as a potent coactivator for IL-2 transcription, and in combination with NFATc, EGR-1 increases transcription of an IL-2 reporter construct 200-fold. Electrophoretic mobility shift assays reveal that recombinant EGR-1 and NFATc bind independently to their target sites within the IL-2 promoter, and the presence of both sites on the same DNA molecule is required for EGR-1·NFATc·DNA complex formation. The transcriptional synergy observed here for EGR-1 and NFATc explains how the abundant nuclear factor EGR-1 contributes to the expression of restrictively expressed genes.

Antigenic activation of resting T cells initiates a cascade of biochemical and metabolic events that lead to cell proliferation, cytokine release, and T cell effector function (1–3). T cell receptor engagement induces sequential signaling reactions that are converted to the nucleus, where transcription of novel genes is induced. Most of the directly activated immediate early genes encode transcription factors that regulate the nuclear events essential for cell proliferation, differentiation, and T cell effector function. In general terms the proteins encoded by the transiently expressed immediate early genes either inactivate immediate early gene transcription in an autoregulatory fashion or they function as transcriptional activators of early gene expression.

The four related early growth response genes *EGR-1* (4 - 6), *EGR-2* (7, 8), *EGR-3* (9, 10), and *EGR-4/pAT133* (11, 12) comprise a family of related immediate early genes. They are coexpressed and coregulated and are transiently induced in distinct cell types in response to mitogens, differentiation, apoptotic signals, and tissue injury (4, 7, 12–15). EGR[1] proteins are closely related within their zinc finger regions; however, their flanking domains are much less conserved. Although all four recombinant EGR proteins have been reported to bind to the same consensus sequences GCG(G/T)GGGCG, binding studies with recombinant EGR-1 and EGR-4/pAT133 proteins expressed in eukaryotic systems revealed different binding affinities and recognition of distinct target sequences (16–18). The EGR-1 protein specifically interacts with the G-rich regulatory zinc finger protein binding site (ZIP) of the human *IL-2* gene promoter, whereas EGR-2, EGR-3, and EGR-4/AT133 do not bind to this promoter element (19). A large number of regulatory GC-rich promoter elements that represent overlapping binding sites for EGR-1 and Sp1 have been recently identified (20–22). As both zinc finger proteins bind alternatively to these elements, it is of interest to define which protein mediates transcriptional regulation through these overlapping binding sites.

The regulation of the *EGR-1* gene is well characterized; however, the functional mechanisms of this protein in gene transcription are less defined. EGR consensus or binding sites are present in promoters of a number of tissue specifically expressed genes such as cytokines, growth factors, and genes involved in cell cycle regulation, *e.g. IL-2*, tumor necrosis factor-α, tumor growth factor-β, insulin-like growth factor, platelet-derived growth factor-α, β chain, *EGR-1*, *EGR-4/pAT133, hox 1.4*, and *nur77* (18–20, 23–28). EGR-1 functions as transcriptional activator, *e.g.* for *ICAM-1* and *CD44* (29–31), as well as a repressor for cellular *CD23* and *Fas/CD95* genes (32). In agreement with this dual function, several activating and one inhibitory domain have been localized within the EGR-1 protein (33, 34). Two factors have been identified that interact with the inhibitory domain and repress EGR-1 transcriptional activity *in vitro* (35, 36). Recently, a synergistic activation of EGR-1 and RelA has been reported in induction of NF-κB1 promoter activity (37).

Within the human *IL-2* gene promoter, we have previously identified a novel regulatory element, termed ZIP, that serves as an overlapping binding region for EGR-1 and Sp1 (19). The ZIP element is located immediately upstream of the distal binding element for the nuclear factor of activated T cells (NFAT). Transfection experiments revealed that both the ZIP site and the distal NFAT site have activating functions (19, 27, 38, 39), and a combination of the ZIP and NFAT elements contribute significantly to *IL-2* gene expression (19).

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[1] The abbreviations used are: EGR, early growth response; NFAT, nuclear factor of activated T cells; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione *S*-transferase; bp, base pair(s).

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NFAT proteins play a key role in cytokine expression, and NFAT binding sites are reported within the promoters of several cytokine genes, *e.g. IL-2, IL-4, TNF-α*, and *GM-CSF* (40–46). In most of these promoters, NFAT binding sites are adjacent to binding sites for AP-1 proteins (*e.g.* in the *IL-2*, *IL-4*, *IL-5*, *CD40L*, and *GM-CSF* gene promoter; reviewed in Ref. 45), and a cooperation of NFAT factors with AP-1 increases the binding affinity to DNA and stabilizes the NFAT·AP-1·DNA complex (47–49). Besides this cooperation with AP-1 proteins, a functional interaction of NFATp with the proto-oncogene c-Maf in transcription of the murine *IL-4* gene has been reported (50).

To characterize which of the two ZIP binding proteins mediates *IL-2* gene transcription, we have analyzed the regulatory role of these zinc finger proteins. Here, we report that the transcription factor Sp1 has no effect on IL-2 transcription but that EGR-1 and NFATc interact synergistically. EGR-1 and NFATc bind independently to adjacent sites within the *IL-2* gene promoter to form an EGR-1·NFATc·DNA complex. Although by itself EGR-1 has little transactivating capacity, this zinc finger protein enhances NFATc transactivation 25-fold. This is the first report that describes a functional interaction between the early growth response protein EGR-1 and a member of the NFAT family of transcription factors.

EXPERIMENTAL PROCEDURES

Plasmids—The reporter plasmid pZNA3-Luc contains three copies of the ZIP, NFAT, and AP-1 binding elements linked to a minimal IL-2 promoter fragment. For construction of pZNA3-Luc, three double-stranded oligonucleotides covering the ZIP·NFAT·AP-1 binding region of the human IL-2 promoter (position −302 to −258) were ligated in head to tail orientation and inserted into plasmid pMILuc4 (19), which contains a minimal IL-2 promoter (position −63 to +51) linked to the firefly luciferase gene. Plasmid pNA3-Luc contains three copies of the NFAT and AP-1 binding sites (position −280 to −258) linked to the minimal IL-2 promoter and the firefly luciferase gene. The sequence of the reporter constructs was confirmed by DNA sequence analysis. Expression plasmid pSG5-EGR1 has a full-length human EGR-1 cDNA inserted (18). The deletion constructs of this expression vector include the DNA binding zinc finger domain and N- or C-terminal parts of the sequence conferring to amino acids 11–435 (ΔC-EGR-1) and 321–543 (ΔN-EGR-1).

Plasmid pPacSp1, which has 2.1 kilobases of the human Sp1 cDNA (encoding the C-terminal 696 amino acids of Sp1) inserted, was kindly provided by Robert Tjian (University of California, Berkeley). Plasmid pSH107c, which contains the full-length human NFATc cDNA (51), was a generous gift of Gerald R. Crabtree (Stanford University, Stanford, CA). The c-Fos and c-Jun expression constructs, pRSVc-fos (52) and pRSV-cJ (53), were obtained from Peter Angel (Forschungszentrum, Karlsruhe). Plasmid pGEX-NFATc, which contains the full-length NFATc cDNA, was kindly provided by E. Serfling (Würzburg, Germany). All plasmids used for transfection were purified by CsCl density gradient centrifugation.

Cell Culture and Transfection—The human helper T cell line Jurkat and 293 kidney cells (kindly provided by Hermann Eibel, University Hospital Freiburg, Germany) were maintained in RPMI 1640 medium (Bio Whittaker), supplemented with 10% heat-inactivated fetal calf serum, penicillin, streptomycin, and fungizone at a density between 0.5×10^5 and 8×10^5 cells per ml. For transient transfections of Jurkat cells, 1×10^7 cells were washed with PBS and resuspended in 0.8 ml of transfection buffer (10 mM glucose, 0.1 mM DTT in RPMI 1640). Cells were transfected by electroporation at 300 V and 960 μF using 5 μg of reporter construct and 7 μg of the indicated expression plasmids. In all experiments, the total amount of transfected DNA was kept constant by addition of pSG5 plasmid DNA. After transfection, cells were incubated for 48 h in culture medium. When indicated, cells were stimulated 24 h after transfection with 1 μg/ml PHA (Murex Diagnostics) and 20 ng/ml PMA (Sigma) for another 24 h. Cells were harvested by washing two times with PBS, lysed by incubation in 250 μl of lysis reagent (Promega) for 8 min at room temperature, and centrifuged for 10 s at maximum speed in an Eppendorf microfuge. Cell supernatant (50 μl) was mixed with 100 μl of luciferase assay reagent (Promega), and the light emission was measured immediately at 25 °C using a luminometer (Berthold Biolumat LB 9500C). The initial 10-s integral of light emission was recorded. All assays were performed in triplicate. Transfection of 293 cells was performed by calcium phosphate precipitation according to standard procedures (54). About 4×10^5 cells were transfected with 2 μg of reporter plasmid and 3 μg of the various expression plasmids, together with 0.5 μg of pRL-TK vector (Promega) to control for transfection efficiency. After 24 h, cells were washed twice with PBS and harvested. Cell lysis and measurement of luciferase activity were performed using the dual-luciferase reporter assay system (Promega).

Expression of Recombinant Proteins—Recombinant EGR-1 protein was expressed in the baculovirus system as described (18). Sf9 cells were infected in Insect-Xpress medium (Bio Whittaker), with recombinant EGR-1 containing virus using a multiplicity of infection of 5. After 3 days, cells were harvested, washed twice in ice-cold PBS, and incubated on ice in hypotonic buffer (10 mM Tris-Cl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM NaF, 0.5 mM DTT, 0.5 mM PMSF, Sigma) for 10 min. Cells were pelleted by centrifugation at 12,000 × *g* for 10 s, and the pellets were incubated on ice for 45 min in lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10 mM NaF, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF). The extracts were cleared by centrifugation at 12,000 × *g* for 20 min at 4 °C and stored in aliquots at −70 °C.

Recombinant NFATc protein was expressed as a GST-fusion protein using plasmid pGEX-NFATc, which contains the full-length NFATc cDNA (40). An overnight culture of pGEX-NFATc transformed cells (DH5α/BL21) was added to 100 ml of LB medium containing 50 μg/ml ampicillin, 0.1% glucose and grown for 3 h at 37 °C. After induction of protein expression by isopropyl-1-thio-β-D-galactopyranoside (0.4 mM), cells were grown at room temperature for another hour. Cells were harvested, resuspended in 2 ml of PBS containing 1% Triton X-100 (Sigma), 0.5 mM DTT, and 0.5 mM PMSF, and lysed on ice by sonication; cellular debris were pelleted by centrifugation. Upon addition of 1 ml of glutathione-agarose beads (1:2 (v/v), Sigma), the suspension was incubated for 20 min at 4 °C on a rotating platform. Beads were collected by centrifugation (1 min at 800 × *g*) and washed five times with PBS (containing DTT and PMSF). GST-NFATc fusion protein was eluted using 50 mM Tris-HCl, pH 8.0, containing 10 mM glutathione (Boehringer Mannheim). Eluted protein was stored in aliquots at −70 °C. Recombinant Sp1 protein was obtained from Promega.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—SDS-polyacrylamide gel electrophoresis was performed as described previously (54, 55) using 10% separating gels and prestained molecular weight marker proteins (Life Technologies, Inc.). Proteins were transferred onto nitrocellulose membrane by semidry blotting (56). Membranes were blocked with 5% (w/v) dried milk in PBS for 30 min and incubated overnight at 4 °C with specific polyclonal rabbit anti-human EGR-1 antiserum (Santa Cruz Biotechnologies) or monoclonal mouse anti-human NFATc antibody (ABR, Inc.).

Electrophoretic Mobility Shift Assays—For electrophoretic mobility shift assays, 1–5 pM double-stranded oligonucleotides were end-labeled with [γ-^{33}P]ATP (specific activity, 3000 Ci/mM; Amersham Buchler). Double-stranded oligonucleotides representing the ZIP site of the human IL-2 promoter (Z; position −302 to −280 relative to the transcription start site), the NFAT site (N; position −280 to −260), as well as the complete IL-2 ZIP·NFAT region (ZN; position −302 to −258) were used. Double-stranded oligonucleotides with the mutated binding sites included ZmN (5′ TGTAT <u>AA</u>CCA CC<u>AA</u>C TTAAA GAAAG GAGGA AAAAC TGTTT CATA 3′), ZNm (5′ TGTAT CCCCA CCCCC TTAAA GAAAG GAGG<u>C</u> AAAAC TGTT<u>G</u> CATA 3′), and ZmNm (5′ TGTAT <u>AA</u>CCA CC<u>AA</u>C TTAAA GAAAG GAGG<u>C</u> AAAAC TGTT<u>G</u> CATA 3′); mutated nucleotides are underlined. Labeled oligonucleotides (10–30 μM) were incubated at 4 °C with recombinant proteins in 20 μl of buffer containing 20 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM DTT, 1 mM $MgCl_2$, and 4% (v/v) Ficoll for 30 min in the presence of 0.5 μg of poly(dI-dC) (Amersham Pharmacia Biotech). In some cases, competitor DNA or specific antiserum directed against EGR-1 (Santa Cruz) or NFATc (ABR) was added to the incubation mixture as indicated. The resulting DNA-protein complexes were separated in a 5% nondenaturing polyacrylamide gel at 4 °C in 0.25 × TBE at 150 V and 20 mA. For competition experiments, the indicated oligonucleotides were used in 200-fold excess.

RESULTS

Regulation of IL-2 Gene Expression—A combination of the ZIP, NFAT, and AP-1 elements of the human *IL-2* gene promoter is important for gene induction. As each element represents binding sites for several transcription factors, we were interested in identifying the factors required for maximal tran-

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TABLE I
Transactivating effects of ZIP, NFAT, and AP-1 binding factors

Transactivating activity of EGR-1, Sp1, AP-1 proteins c-Fos and c-Jun, and NFATc on reporter construct pZNA3-Luc, which contains three copies of the ZIP · NFAT · AP-1 region of the human *IL-2* gene promoter linked to a minimal *IL-2* gene promoter and to the firefly luciferase gene, is shown.

Expression plasmid	Relative light units (± S.D.)	Fold induction
None	105 (± 10)	1.0
EGR-1	141 (± 43)	1.3
Sp1	119 (± 86)	1.1
AP-1 (c-Fos, c-Jun)	296 (± 65)	2.8
NFATc	854 (± 73)	8.1
EGR-1 + NFATc	21,460 (± 2,192)	204.4
Sp1 + NFATc	870 (± 739)	8.3
NFATc + AP-1	3,442 (± 1,562)	32.8
EGR-1 + NFATc + AP-1	19,537 (± 12,900)	186.1

scription. To this end, cotransfection experiments were performed with various expression vectors and with reporter construct pZNA3-Luc containing three copies of the ZIP·NFAT·AP-1 region linked to a minimal *IL-2* gene promoter and the firefly luciferase gene. The zinc finger proteins Sp1 and EGR-1 showed little effect when used as single proteins (1.1- and 1.3-fold induction), AP-1 proteins (c-Fos and c-Jun) showed a weak activating capacity (2.8-fold induction), whereas NFATc displayed a strong activating effect on reporter gene transcription (8.1-fold) (Table I and Fig. 1*A*). The transcriptional activity observed with NFATc is in agreement with previous results (39) and underlines the important role of this protein in *IL-2* gene induction. Similar results were obtained with a reporter construct that lacks the ZIP site but has three copies of the NFAT·AP-1 sites linked to the minimal *IL-2* gene promoter (pNA3-Luc) (Fig. 1*B*).

EGR-1 but Not Sp1 Interacts with NFATc—As previous experiments demonstrated that a combination of the ZIP- and NFAT elements induces strong IL-2 promoter activity (19), we asked whether factors binding to these adjacent promoter sites do interact. The transcriptional activity obtained by a combination of two proteins is shown as fold induction compared with the activity obtained with the NFATc expression vector, which was set to 1. A combination of Sp1 and NFATc showed no enhancing effect (Fig. 2 and Table I); however, EGR-1 potentiated NFATc transcription and increased transactivation 25-fold (Fig. 2*A* and Table I). Although both Sp1 and EGR-1 bind to the same promoter element, EGR-1 but not Sp1 cooperates with NFATc in *IL-2* gene induction.

To test whether this cooperation is dependent on the DNA binding sites, a reporter plasmid (pNA3-Luc) was used that contains the NFAT and AP-1 binding sites but lacks the ZIP site. The presence of the ZIP element is essential for the observed interaction, as in the absence of an EGR binding site, EGR-1 affected NFATc activity only 2.3-fold (Fig. 2*B*). This reduced effect suggests that binding to DNA is a prerequisite for the functional interaction of the two nuclear factors.

The AP-1 Proteins (c-Fos and c-Jun) Do Not Affect the Transcriptional Synergy between EGR-1 and NFATc—As AP-1 proteins are reported to interact with NFAT factors (57, 58), we asked whether the AP-1 proteins c-Fos and c-Jun do influence the functional synergy between EGR-1 and NFATc. Transfection experiments with reporter plasmid pZNA3-Luc confirmed the previously described interaction of AP-1 with NFATc, as a combination of c-Fos and c-Jun increased the transcriptional activity of NFATc 4-fold (Fig. 2*C* and Table I). Although c-Fos and c-Jun increased NFATc activity, the AP-1 factor failed to further enhance the synergistic interaction of EGR-1 and NFATc. In contrast, a slight decrease was observed when all four proteins (EGR-1, NFATc, c-Fos, and c-Jun) were simultaneously expressed (Fig. 2*C* and Table I).

The observed interaction of AP-1 proteins c-Fos and c-Jun with NFATc was also observed with reporter construct pNA3-Luc, which lacks the ZIP site. In the absence of an EGR-1 binding site, AP-1 proteins influenced the transcriptional activity of NAFTc to a similar extent (Fig. 2*D*).

Activity of Truncated Constructs—Several activating domains and one inhibitory domain have been identified within the N-terminal part of the EGR-1 protein (33, 34). Given this modular composition, we tried to localize the domain responsible for the functional interaction with NFATc. To this end, a vector encoding a truncated EGR-1 protein with the N-terminal 320 amino acids deleted (ΔN-EGR-1) was transfected together with NFATc. This truncated protein retained the activity of wild-type EGR-1 almost completely (Fig. 3). The interaction with NFATc was further enhanced upon transfection with an EGR-1 deletion mutant that lacked the complete C-terminal 212 amino acids (ΔC-EGR-1). This truncated form of EGR-1 increased NFATc activity about 60-fold, thus showing a 2.5-fold stronger activity than the wild-type protein (Fig. 3). These results show independent contribution of both the N- and the C-terminal domains of EGR-1 in the observed interaction with NFATc. They also indicate that a repressor domain is located within the C-terminal region.

Relevance for IL-2 Gene Induction—Having demonstrated a functional synergy between the two nuclear factors EGR-1 and NFATc, we were interested in defining whether this interaction is of physiological relevance. We therefore tested whether a combination of the two proteins induces a wild-type IL-2 promoter construct that contains all regulatory sites relevant for induction in T cells. The corresponding reporter construct of the *IL-2* gene promoter (plasmid pCILuc 1, ranging from –366 to +51; Ref. 19) has several positive and negative regulatory regions (59) linked to the firefly luciferase gene. Expressed as single factors, neither EGR-1 nor NFATc activated transcription of this complete *IL-2* gene promoter in Jurkat T cells (Fig. 4). The different effects of NFATc on this wild-type promoter compared with the synthetic promoter construct (Fig. 1) demonstrates that the additional regulatory elements located downstream of the distal NFAT site are important for IL-2 promoter activity. A combination of both transcription factors induced activation of this wild-type construct almost 10-fold. This level of activation reached 36.8% of the activity obtained after stimulation with the potent mitogens PHA and PMA (Fig. 4).

IL-2 Induction in Non-lymphoid Cells—IL-2 gene expression is restricted to lymphoid organs and lymphoid cells and is not detectable in other tissues or in non-lymphoid cells. We therefore were interested to analyze whether EGR-1 and NFATc are sufficient to induce *IL-2* reporter gene expression in non-T cells. We chose human 293 kidney cells that do not express the *IL-2* gene to further analyze the role of these two proteins on *IL-2* reporter gene activity. In 293 cells, NFATc but not EGR-1 induced the reporter construct. EGR-1 acted as a coactivator for NFATc activity, and in combination the two proteins induced reporter gene activity 60-fold (Fig. 5*A*). Similar effects were also observed in non-lymphoid, non-human Chinese hamster ovary cells (data not shown).

Recombinant Expression of EGR-1 and NFATc—The functional interaction between EGR-1 and NFATc demonstrated in T cells and in non-lymphoid cells prompted us to analyze whether the two proteins interact directly and form heterodimeric complexes. Recombinant EGR-1 was expressed in the baculovirus system as an 80-kDa protein (18), and NFATc was expressed as a 105-kDa GST-fusion protein in *Escherichia*

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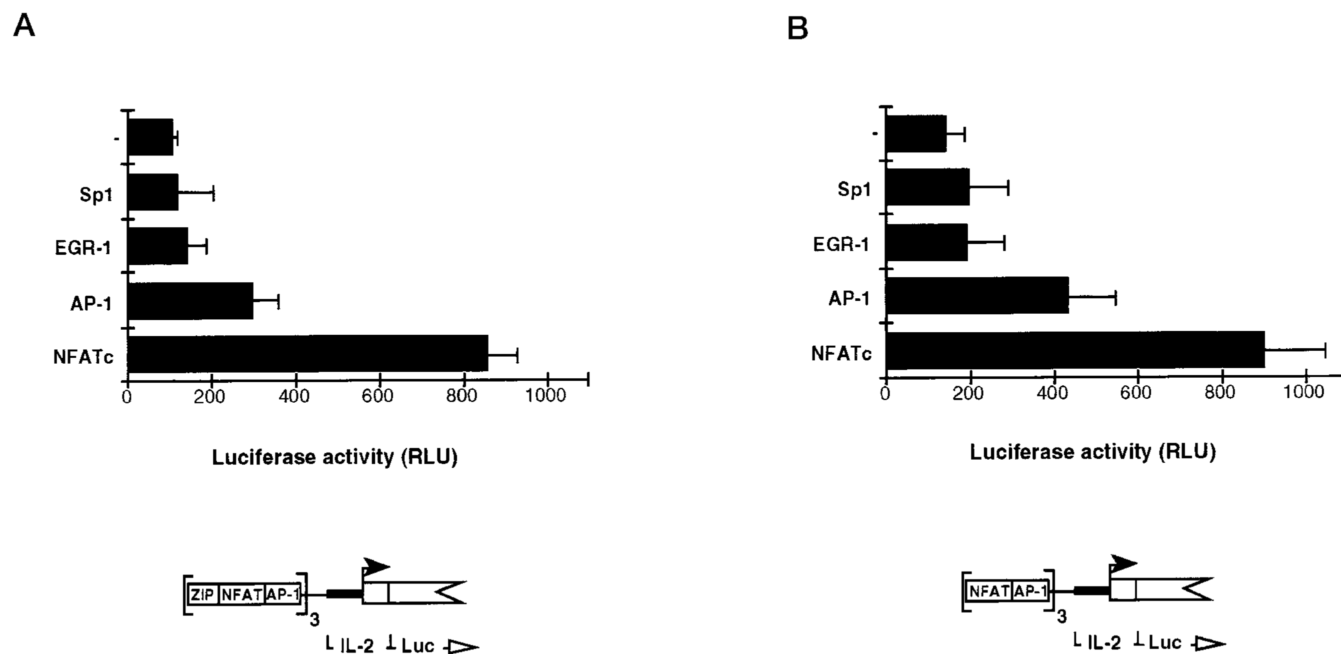

FIG. 1. **Transcriptional effects of single factors binding to the ZIP·NFAT·AP-1 element of the *IL-2* gene promoter.** *A*, Jurkat cells were transfected with reporter construct pZNA3-Luc containing three copies of the ZIP·NFAT and AP-1 binding element linked to a minimal *IL-2* gene promoter and the luciferase gene together with expression vectors coding for Sp1, EGR-1, AP-1 (c-Fos and c-Jun), and NFATc. *B*, the reporter construct pNA3-Luc, lacking the ZIP site but containing three copies of NFAT and AP-1 binding elements linked to a minimal *IL-2* gene promoter and the luciferase gene, was cotransfected with expression vectors coding for the indicated proteins. Each column represents the mean of at least five independent experiments; the mean value and standard deviations are shown. A schematic representation of the reporter constructs with the indicated binding sites, the minimal *IL-2* gene promoter (position −63 to +51) and the firefly luciferase gene (Luc), is shown at the bottom of each panel.

coli as described (Ref. 40 and data not shown).

Recombinant EGR-1 and NFATc Bind to the Human IL-2 Gene Promoter—Both recombinant proteins bind specifically to either the ZIP- or the NFAT site and form single protein-DNA complexes (Fig. 6*A*, *lanes 1* and *4*). An additional DNA-protein complex was formed when the two specific binding sites were combined on one oligonucleotide (Fig. 6*B*, *lane 3*, complex III). Based on competition experiments with unlabeled oligonucleotides (Fig. 6*B*, *lanes 4–6*) and with mutated oligonucleotides (data not shown), binding was specific. Complexes I and III were competed by a ZIP-containing oligonucleotide (Z) (Fig. 6*B*, *lane 4*), whereas complexes II and III were competed by an NFAT oligonucleotide (N) (Fig. 6*B*, *lane 5*). All three complexes were competed with an oligonucleotide containing both binding sites (ZN) (Fig. 6*B*, *lane 6*). These data demonstrate specific binding of recombinant EGR-1 and NFATc, and they also show that EGR-1 and NFATc bind simultaneously within the *IL-2* gene promoter.

Identification of the Protein Complexes by Antiserum—The identity of the protein complexes was further confirmed by the use of specific antisera. Antiserum against EGR-1 affected mobility of complex I, resulting in formation of complexes with reduced mobility (complex IV) (Fig. 6*C*, *lane 2*). In the presence of NFATc, the same antiserum affected the mobility of complexes I and III but did not influence the NFATc complex (complex II) (Fig. 6*C*, *lane 4*). This result clearly demonstrates that EGR-1 is part of complex III. Similar results were observed using NFATc antiserum. This antiserum affected binding of NFATc (complex II) (Fig. 6*D*, *lane 1*) and resulted in formation of a supershift (Fig. 6*D*, *lane 2*, complex IV). Binding of EGR-1 was not affected with this antiserum (Fig. 6*D*, *lane 4*). The mobility of the supershifted complex is similar to that of the EGR-1·NFAT-containing complex, and the two bands could only be separated under very stringent conditions (data not shown). In summary, competition analysis and the use of specific antisera confirmed that complex III represents an EGR-1·NFATc·DNA complex.

EGR-1 and NFAT Bind DNA Independently and Do Not Physically Interact in the Absence of DNA—Within the human *IL-2* gene promoter, the ZIP and the NFAT element are directly adjacent to each other. We therefore asked (i) whether the two proteins bind simultaneously, (ii) whether binding of one factor prevents binding of the other, or (iii) whether in a DNA-bound configuration the bound protein enhances binding of the second factor. As EGR-1, NFATc, and EGR-1·NFATc complexes are easily identified by electrophoretic mobility shift assays, we chose this system to analyze whether the two proteins form protein-protein complexes when one protein is bound to DNA. The mobility of EGR-1 bound to an oligonucleotide containing a single ZIP site was not influenced by recombinant NFATc, and no additional complex was detected (Fig. 7, *lane 3*). Similarly, EGR-1 did not affect the mobility of NFATc bound to an oligonucleotide with an NFAT site (Fig. 7, *lane 6*). Simultaneous binding of NFATc and EGR-1 was only detected when both target sequences were located on the same oligonucleotide (Fig. 7, *lane 9*). These data demonstrate that EGR-1 and NFATc do not form heterodimeric protein-protein complexes but are brought together by their specific binding sites on one promoter element.

Sp1 Binding to the ZIP Element—To prove that the ZIP site is also a binding site for the Sp1 zinc finger protein and that the formation of the slower mobility complex is independent of EGR-1·NFAT interaction, we performed binding studies with recombinant Sp1. Sp1 bound to the ZIP site (Fig. 8*A*, *lane 1*, complex IV). Upon binding to separate oligonucleotides, only single Sp1 and NFATc protein-DNA complexes were detected (*lane 4*). However, upon binding to one oligonucleotide that includes both binding sites, an additional complex (V) of slower mobility was detected (*lane 6*). The mobility of this Sp1·NFATc complex is different from that of the EGR-1·NFATc complex

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FIG. 2. **Effect of the ZIP binding zinc finger proteins Sp1 and EGR-1 in regulation of NFATc and AP-1 transcriptional activity.** *A*, Jurkat cells were transfected with reporter construct pZNA3-Luc and a combination of expression vectors coding either for Sp1 and NFATc or EGR-1 and NFATc. *B*, a combination of EGR-1 and NFATc expression vectors was transfected together with reporter construct pNA3-Luc containing three copies of NFAT and AP-1 binding elements but lacking the ZIP site. *C*, Jurkat cells were transfected with the reporter construct pZNA3-Luc in combination with the indicated expression vectors. The c-Fos and c-Jun vectors were transfected to form the AP-1 factor. *D*, the same combinations of expression vectors were transfected together with the reporter construct pNA3-Luc containing three copies of NFAT and AP-1 binding elements but lacking the ZIP site. Values represent fold induction compared with the activity obtained by transfecting NFATc alone, which was set to 1. Each *column* represents the mean of at least five independent experiments, and standard deviations are indicated by the *bars*.

(complex III, *lane 7*). Due to the increased length of the ZN oligonucleotide, the mobility of single protein complexes is different (*e.g.* compare *lane 1* or *4* with *lane 6*).

To further confirm the specificity of the slow mobility complex, mutant forms of the Il-2 promoter elements were used in gel shift assays, which had either a single binding site mutated (ZmN and ZNm) or had both sites mutated (ZmNm). Binding analysis confirmed that the mutations abrogated binding of the corresponding protein, and in all cases no additional third complex of slower mobility was detected (Fig. 8*B*).

DISCUSSION

The *EGR-1* gene is induced in a wide variety of cells by a number of distinct agents and links biochemical signaling to gene transcription. Little is known how this abundantly expressed zinc finger protein regulates tissue-specific gene expression. We have analyzed the function of EGR-1 in *IL-2* gene induction, as transcription of this cytokine gene is highly restricted to lymphoid cells (40, 41). Within the IL-2 promoter, EGR-1 binds to the regulatory ZIP element, which contributes significantly to IL-2 transcription. The ZIP element, which serves as an overlapping binding site for EGR-1 and the constitutively expressed transcription factor Sp1 (19), is located immediately upstream of the distal NFAT·AP-1 binding site. This NFAT·AP-1 element is important for *IL-2* gene transcription, and at this site NFAT factors interact with AP-1 proteins

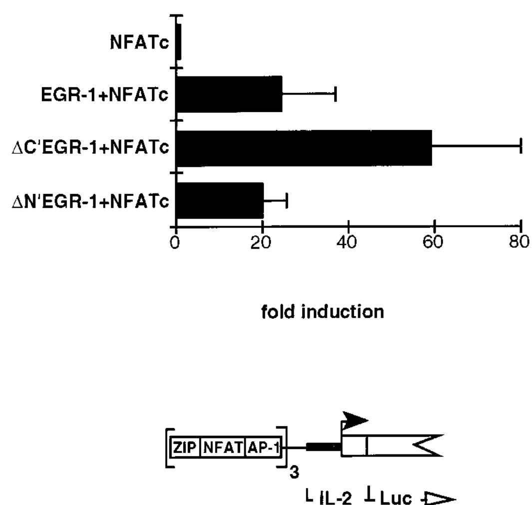

FIG. 3. **Effect of N- and C-terminal deletions of EGR-1 on the functional interaction with NFATc.** N- or C-terminal domains preceding or following the zinc finger domain of the EGR-1 protein were deleted, and the interaction of these truncated proteins with NFATc on constructs pZNA3-Luc was analyzed in Jurkat T cells. The activity obtained with the NFATc protein was set to 1, and the fold induction obtained with the wild-type protein or the truncated proteins is shown. Each *column* represents the mean of at least five independent experiments, and standard deviations are indicated by the *bars*.

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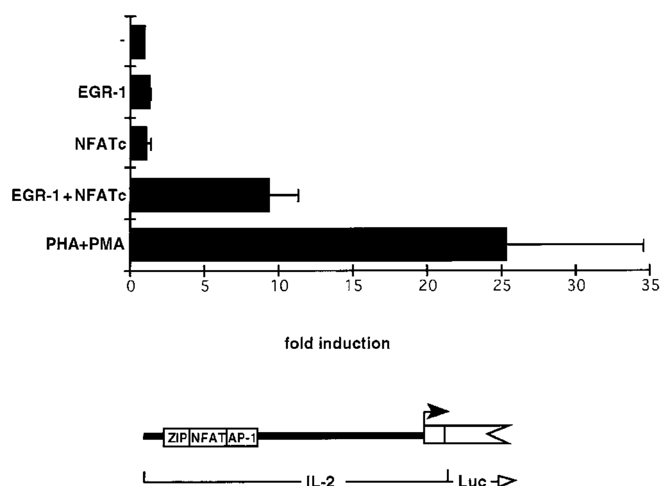

FIG. 4. **Synergistic interaction of EGR-1 and NFATc mediates transcription of the wild-type *IL-2* promoter in Jurkat cells.** Jurkat cells were transfected with a reporter construct containing a 366-bp fragment of the human *IL-2* gene promoter fused to the firefly luciferase gene (Luc) together with expression vectors coding for EGR-1, NFATc, or a combination of EGR-1 and NFATc proteins. In addition, the synergistic effect of EGR-1 and NFATc is compared with the promoter activity obtained by stimulation of the transfected IL-2 promoter construct by PHA and PMA. The values show fold induction compared with the luciferase activity obtained with the reporter construct in untreated cells. Each *column* represents the mean of at least five independent experiments, and standard deviations are indicated by the *bars*. A schematic representation of the reporter construct is shown at the bottom.

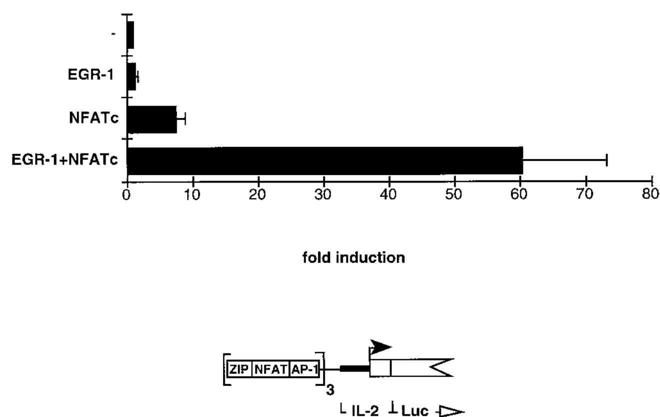

FIG. 5. **Synergistic interaction of EGR-1 and NFATc in nonlymphoid cells.** Human 293 kidney cells were transfected with reporter construct pZNA3-Luc together with expression vectors coding for EGR-1, NFATc, or a combination thereof. The transcriptional activity is shown as fold induction of the reporter construct. Each *column* represents the mean of at least three independent experiments, and standard deviations are indicated.

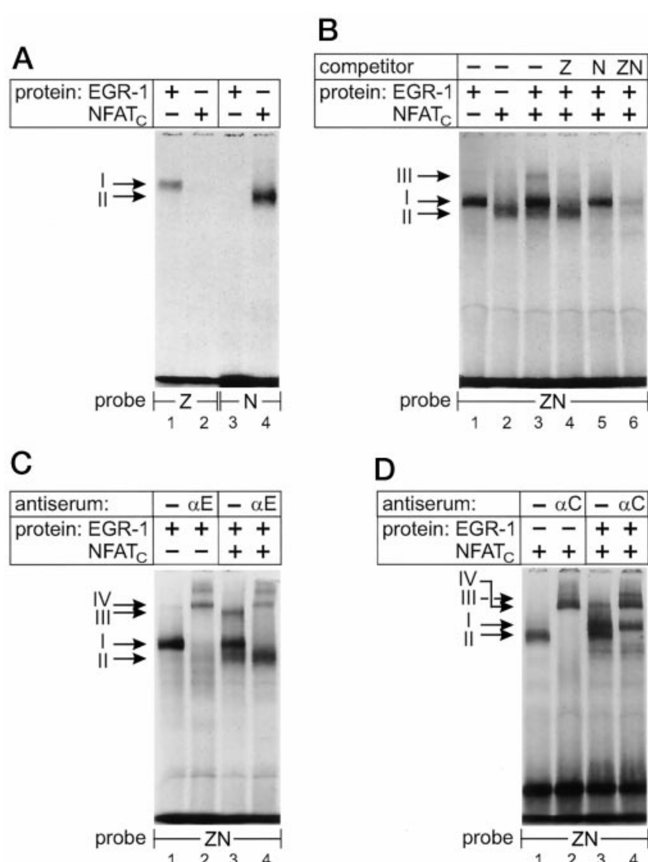

FIG. 6. **Specific binding of EGR-1 to the ZIP and NFATc protein to the NFAT region of the human *IL-2* gene promoter.** *A*, binding of recombinant EGR-1 to a ^{33}P-labeled ZIP oligonucleotide (Z) results in formation of a specific retarded complex (I) (*lane 1*). The same protein does not bind to a labeled oligonucleotide representing the NFAT element (N) of the human *IL-2* gene promoter (*lane 3*). Binding was assessed by electrophoretic mobility shift assay. The recombinant NFATc protein binds to the NFAT site (complex II) (*lane 4*) but not to the ZIP site of the *IL-2* gene promoter (*lane 2*). *B*, binding of recombinant EGR-1 and NFATc to a ^{33}P-labeled oligonucleotide that represents the ZIP and the NFAT site of the human *IL-2* gene promoter (ZN). In addition to the single protein-DNA complexes (I and II), simultaneous binding of both proteins results in formation of a third complex (III). Competition with either the ZIP (*lane 4*) or the NFAT oligonucleotide (*lane 5*) results in disappearance of the complex III together with either complex I (*lane 4*) or complex II (*lane 5*). Oligonucleotides that contain both binding sites compete all three complexes (*lane 6*). *C*, effect of antiserum against EGR-1. Anti-EGR-1 (αE) affects mobility of complex I (*lanes 2* and *4*) and of complex III (*lane 4*), but not of the NFATc containing complex II (*lane 4*), and results in the appearance of a new complex with lower mobility (complex IV). *D*, antiserum directed against NFATc (αC) affects mobility of complex II (*lanes 2* and *4*) and also of complex III (*lane 4*) but does not affect mobility of complex I (*lanes 2* and *4*). As the mobility of the complexes III and IV are almost identical, the disappearance of the EGR-1·NFATc containing complex III was not detected in this particular experiment.

(40, 48, 49). Transfection experiments suggest a cooperation between factors binding to the ZIP, NFAT, or AP-1 elements. Here, we demonstrate that EGR-1, but not Sp1, mediates IL-2 transcription via the ZIP site by functioning as a potent coactivator for the transcription factor NFATc. Binding studies with recombinant proteins show that the two factors bind independently to the adjacent promoter sites and form complexes when their binding sites are present on the same DNA molecule.

Due to its tissue-specific expression and its essential role in immune response, regulation of the human *IL-2* gene is extensively analyzed. Initial studies have shown that a 300-bp promoter fragment confers tissue specificity and inducibility in T cells (38, 39); however, additional regulatory elements have been described further upstream (60). Several regulatory elements representing binding sites for distinct transcription factors have been identified within the 300-bp promoter element (40). A critical role was reported for a regulatory domain around position −290 that turned out to be a binding site for a combination of NFAT and AP-1 proteins (38, 39). Four distinct members of the NFAT family are identified, and NFATc and NFATp are considered to play a key role in cytokine gene expression (45, 46). Adjacent NFAT and AP-1 binding sites are common in cytokine gene promoters (*e.g. IL-2*, *IL-4*, *IL-5*, *GM-CSF*, *IFN-γ*, and *CD40L*) and a cooperation between these nuclear proteins has been reported in terms of DNA binding, stability of the DNA-bound complex, and transactivating functions (49, 57).

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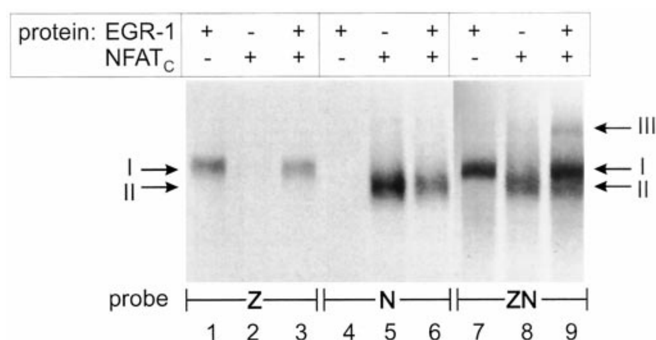

FIG. 7. **Complex formation of EGR-1 and NFATc requires linkage of the binding sites to one DNA molecule.** Recombinant EGR-1 binds specifically to the ZIP site complex I (*lane 1*) (Z) but not to the NFAT site (N) (*lane 4*), whereas NFATc binds to the NFAT site complex II (*lane 5*) but not to the ZIP site (*lane 2*). Complex formation of EGR-1 and NFATc is not detectable when both proteins are added and a single binding site is provided (either the ZIP site or the NFAT site) (*lanes 3* and *6*). Complex formation and simultaneous binding of the two recombinant proteins is detectable when both the ZIP and the NFAT sites (ZN) are located on the same oligonucleotide (*lane 9*).

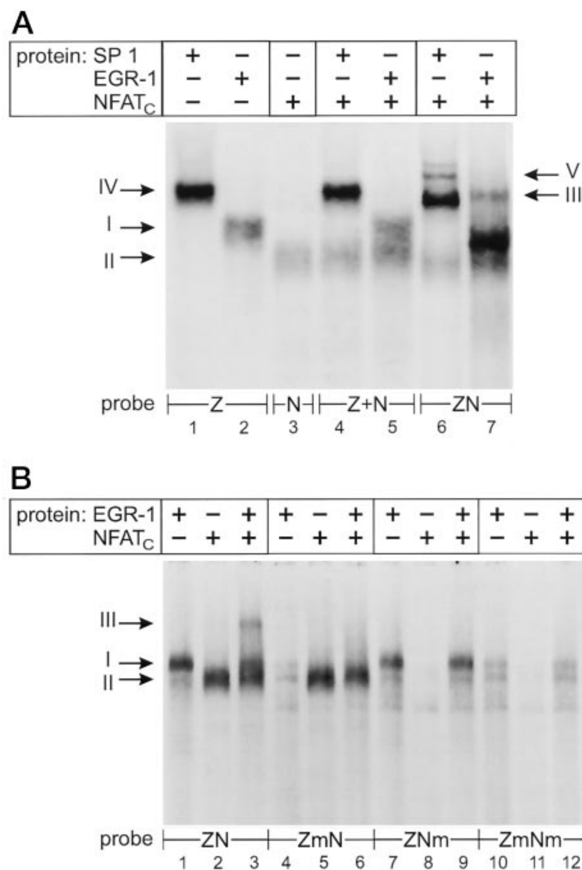

FIG. 8. **Binding of Sp1 to the ZIP element and elimination of complex formation by mutation of the binding sites.** *A*, the zinc finger protein Sp1 binds to the ZIP element (Z) (*lane 1*). Specific complexes were detected when oligonucleotides with single binding sites were used (*lanes 2–5*): complex IV, Sp1 (*lane 1*); complex I, EGR-1 (*lanes 2* and *5*); and complex II, NFATc (*lanes 3–5*). Additional complexes of slower mobility were detected when both the ZIP and the NFAT binding sites were present on the same oligonucleotide (complex III and complex V; *lanes 6* and *7*). The different mobility of Sp1 bound to the ZIP-NFAT sequence (ZN) compared with the single ZIP site (Z) (compare *lanes 1*, *4*, and *6*) is explained by the different length of these oligonucleotides. *B*, formation of complex III requires intact binding sites on the same oligonucleotide (*lane 3*). Double-stranded oligonucleotides, which have either one binding site (ZmN or ZNm) or both sites mutated (ZmNm), were used for binding analysis with Egr-1 and NFATc. Mutation of the ZIP site abrogated binding of EGR-1 (*lanes 4*, *6*, *10*, and *12*), whereas mutation of the NFAT site abrogated NFATc binding (*lanes 8*, *9*, *11*, and *12*).

Within the human *IL-2* gene promoter, the distal NFAT site is directly flanked by the regulatory ZIP element. Transfection assays revealed that a combination of the ZIP and the distal NFAT element interact in a synergistic fashion and contribute substantially to *IL-2* gene transcription (19). The synergistic effect observed for these adjacent promoter elements is mediated by the nuclear factors EGR-1 and NFATc but not by Sp1. In electrophoretic mobility shift assay studies, both EGR-1 and NFATc form specific DNA-protein complexes and bind simultaneously to an oligonucleotide that reflects the native IL-2 promoter. Unlike other interacting transcription factors, the two proteins lack prominent domains typical for protein-protein interaction, and they do not form homo- or heterodimeric protein-protein complexes (data not shown). Thus, we conclude that binding of EGR-1 and NFATc is independent, and apparently each of the two proteins does not influence binding of the partner protein (Figs. 6 and 7). Independent binding of the two proteins is further confirmed by transfection experiments, as the coactivator function of EGR-1 requires the presence of a ZIP site (Fig. 2).

The close proximity of the ZIP-, NFAT-, and AP-1 sites suggests a direct cooperation of all three proteins in gene transcription. Previous studies have reported a regulatory role of AP-1 proteins on IL-2 transcription and a cooperation between the AP-1 proteins c-Fos and c-Jun and NFATp. NFATp·AP-1 cooperation increases the stability of the NFAT-DNA complexes about 20-fold, and AP-1 protein influences the transcriptional activity of NFATp (49, 57). Our data confirm an interaction of the AP-1 proteins c-Fos and c-Jun with NFAT proteins, as c-Fos and c-Jun increase NFATc transcriptional activity 4-fold (Fig. 2). Although AP-1 regulates NFAT binding and transcription, apparently this factor fails to further enhance the synergy of EGR-1 and NFATc.

In addition to the ZIP and the distal NFAT site, several regulatory elements and binding factors have been defined within the *IL-2* gene promoter (40, 41). A negative regulatory element seems important for repression of *IL-2* gene transcription (61), and expression of this inhibitory factor in unstimulated Jurkat T cells may explain the lack of NFATc activity on the intact *IL-2* gene promoter (Fig. 5). Interestingly, the synergy of EGR-1 and NFATc induces a reporter construct containing the complete 366 bp of the human *IL-2* promoter in T cells. Thus, the activating capacity of the two factors can overrule the action of repressive factor(s) that function in unstimulated Jurkat cells.

The exact mechanisms of the synergistic interaction between EGR-1 and NFATc are currently unknown. A similar functional synergy has recently been reported for EGR-1 and Rel A, another member of Rel protein family (37). Apparently, EGR-1 does not form physical complexes with NFATc or with RelA. However, upon binding to the *IL-2* gene promoter, EGR-1 and NFATc are in close proximity, and the proteins may even contact each other. The use of deletion mutants confirmed the existence of positive and negative regulatory domains within the EGR-1 protein (Fig. 3) (34–36). The functional assay shows that both the N- and the C-terminal deletion mutants act independently as strong coactivators in NFATc-mediated transcription (Fig. 3). The even stronger activity of the C-terminal deletion mutant of EGR-1 indicates the existence of an additional repressor domain located within the C-terminal part of the protein. The regulatory domains–potentially the activation domains– of the two proteins may form a cluster that may recruit additional factors relevant for gene activation. This recruited factor(s) may represent part of the general transcrip-

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tion complex. The weaker effect observed for EGR-1 and NFATc interaction in non-lymphoid 293 cells and Chinese hamster ovary cells (60- and 30-fold) (Fig. 4 and data not shown) compared with Jurkat T cells (204-fold induction; Table I) suggests a restricted expression of this recruited factor(s).

The EGR-1 protein is one of the factors that links common biochemical signaling pathways to regulation of gene expression. As demonstrated here for the *IL-2* gene promoter, EGR-1 plays a central role in inducible gene transcription. By synergistic interaction with a restrictively expressed transcription factor, the ubiquitously induced EGR-1 protein contributes to expression of the *IL-2* gene in T lymphocytes. The large number of gene promoters containing EGR-1 target sites and in particular the increasing number of promoters with overlapping EGR-1- and Sp1 sites (21, 22, 62) indicates for EGR-1 a general and important role in gene transcription. To further define the transcriptional role of EGR-1 in tissue-specific gene expression it will be of great interest to identify the mechanisms of interaction with transcription factors like NFATc.

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