

Transcriptional Regulation of the *Icam-1* Gene in Antigen Receptor- and Phorbol Ester-stimulated B Lymphocytes: Role for Transcription Factor EGR1

By Jonathan S. Maltzman, Julie A. Carman, and John G. Monroe

From the Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Summary

Intercellular adhesion molecule (ICAM) 1/CD54 plays an important role in T cell-dependent B cell activation and for function of B lymphocytes as antigen-presenting cells. ICAM-1 expression is upregulated as a consequence of B lymphocyte antigen receptor (BCR) signaling, thereby serving to render antigen-stimulated B cells more receptive to T cell-mediated costimulatory signals. We have investigated BCR-induced expression of the *Icam-1* gene in primary B cells and B cell lines and have found it to be dependent on BCR-induced expression of the transcription factor EGR1. *Icam-1* transcription, induced by BCR cross-linking or bypassing the BCR with phorbol ester, is absent in a B cell line in which the EGR1-encoding gene (*egr-1*) is methylated and not expressed. A potential EGR1-binding site was located at -701 bp upstream of the murine *Icam-1* gene transcription start site and shown by electrophoretic mobility shift assay to bind to murine EGR1. Mutation of this site in the context of 1.1 kb of the *Icam-1* promoter significantly abrogated transcriptional induction by phorbol ester and anti- μ stimulation in primary B cells. A direct effect of EGR1 on the *Icam-1* promoter is suggested by the ability of EGR1 expressed from an SV40-driven expression vector to transactivate the wild-type *Icam-1* promoter, whereas mutation of the EGR1 binding motif at -701 bp markedly compromises this induction. These data identify EGR1 as a signaling intermediate in BCR-stimulated B cell functional responses, specifically linking BCR signal transduction to induction of the *Icam-1* gene. Furthermore, similar findings for BCR-induced CD44 gene induction (Maltzman, J.S., J.A. Carman, and J.G. Monroe. 1996. Role of EGR1 in regulation of stimulus-dependent CD44 transcription in B lymphocytes. *Mol. Cell. Biol.* In press) suggest that EGR1 may be an important signaling molecule for regulating levels of migration and adhesion molecules during humoral immune responses.

Antigen-specific activation of B lymphocytes is a complex process initiated by signals generated through the B cell antigen receptor (BCR)¹. BCR signaling involves a series of increasingly well-defined biochemical events resulting in alterations of gene expression and subsequent changes in the phenotypic and activation state of the B cell (1). Although necessary to initiate the B cell response, in most cases BCR-generated signals are insufficient to drive later activation events such as proliferation and differentiation into antibody-secreting cells (2). For these later events, antigen-stimulated B cells require contact-dependent signals delivered by T cells. These contact-dependent second signals involve ligand-receptor interactions between molecules expressed on the antigen-stimulated T and B cells (2).

Molecules on the B cell that may be involved in costimulation with BCR signals include CD40 and MHC class II antigens, which interact with T cell surface proteins CD40 ligand (gp39) and TCR/CD4, respectively (3, 4). Additional paired proteins on the antigen-activated B and T cells function primarily to stabilize the physical interaction between these cells. These adhesion molecules may initiate some costimulatory function as well (5, 6). One of these cell adhesion molecules is intercellular adhesion molecule (ICAM) 1/CD54.

ICAM-1 is the prototypic member of a family of ligands for the β -2 integrin LFA-1. This family also includes ICAM-2 and ICAM-3, although ICAM-1 is thought to play the predominant role in B cell-T cell interactions (7, 8). Besides mediating interactions between B and T cells, ICAM-1-LFA-1 interactions have also been implicated in B lymphocyte homotypic adhesion (9). Expression of ICAM-1 on B lymphocytes is upregulated by stimulation. For example, stimulation of B lymphocytes by BCR cross-

¹Abbreviations used in this paper: BCR, B cell antigen receptor; CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM-1, intracellular adhesion molecule 1; PAP, placental alkaline phosphatase.

linking results in an increase in cell surface ICAM-1 levels (11, 12). In endothelial cells, induced increase in ICAM-1 expression is the result of elevated transcription of the *Icam-1* gene (13, 14).

Although transcriptional induction of the *Icam-1* gene by BCR cross-linking can be inferred from other studies (11, 12), the molecular processes involved in linking this gene to antigen receptor-initiated signaling events have not been analyzed, nor have the transcriptional control elements for *Icam-1* in B cells been studied. Analysis of the human *Icam-1* promoter revealed a number of potential binding sites for inducible and constitutively expressed transcription factors, including NF- κ B, SP1, and EGR1. In this study, we provide evidence that a B cell line that lacks EGR1 expression is also compromised in its ability to upregulate *Icam-1* gene expression, suggesting a role for the transcription factor EGR1 in BCR-induced *Icam-1* expression.

EGR1 (NGFI-A [15], Krox-24 [16], *zif268* [17], TIS8 [18]) is encoded by an immediate-early response gene and is induced rapidly and transiently (within 30 min and maximally by 2 h) in response to BCR cross-linking or treatment with phorbol esters (19). Protein expression is maximal by 4 h after PMA or anti- μ stimulation (19). By comparison, phorbol ester- and BCR-dependent increases in ICAM-1 protein expression are delayed, detectable by FACS[®] analysis by 6 h after stimulation (12). EGR1 has been shown to be a transcriptional activator both in lymphocytes (20) and other cell types (21, 22). A growing number of EGR1-regulated genes in fibroblasts, neurons, and kidney have been described (23–26). Interestingly, although *egr-1* expression is a common component of the inducible immediate-early gene response in nearly all cells studied, the majority of the EGR1-regulated genes so far identified have been tissue specific or restricted (27, 28).

Based on the association of EGR1 with BCR activation (19), its transcriptional regulatory activity (20–22), the kinetics of its expression relative to *Icam-1*, and our preliminary studies in EGR1-expressing and -nonexpressing B cells, we considered EGR1 as a possible regulator of *Icam-1* induction after BCR cross-linking. This hypothesis was tested in these studies.

Materials and Methods

Isolation of Murine B Lymphocytes, Cell Culture, and Flow Cytometry. The WEHI-231 B cell lymphoma (American Type Culture Collection, Rockville, MD) and all limiting dilution subclones including WEHI-231.7 and WEHI-231.1F1 were maintained in DME, high glucose supplemented with 10% FCS (Hyclone, defined; Hyclone Laboratories Inc., Logan, UT), SerExtend (Hana Biologicals, Berkeley, CA), 2 mM L-glutamine, nonessential amino acids (GIBCO/BRL, Gaithersburg, MD), and 5×10^{-5} M 2-ME. Cells were maintained at a minimum and maximum density of 10^4 and 5×10^5 /ml, respectively.

B lymphocytes were isolated from spleens of BALB/c mice as previously described (29). Briefly, spleens were ground between the frosted ends of two glass slides to produce single cell suspensions. After depletion of T cells by treatment with anti-Thy1.2 antibody and complement and red blood cells by lysis with Gey's

solution, the remaining cell suspension was centrifuged over a 75% Percoll cushion, and the interface was collected. This treatment results in a population of $>90\%$ IgM⁺B220⁺ cells. Cells were then incubated at 37°C, 5% CO₂, for 72 h in B cell assay media (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, nonessential amino acids, 100 μ g/ml penicillin, 100 μ g/ml streptomycin, and 5×10^{-5} 2-ME) and 50 μ g/ml LPS (Sigma Chemical Co., St. Louis, MO).

Flow cytometric analysis was carried out exactly as described (30) using FITC anti-mouse μ F(ab')₂ (PharMingen, San Diego, CA) on a FACScan flow cytometer with LYSIS II software (Becton Dickinson & Co., Mountain View, CA).

Plasmids and Constructs. pBlueICAM containing the full-length ICAM-1 cDNA in the EcoRI site of pBluescript SK⁺ (Stratagene Inc., La Jolla, CA) was provided by Dr. A. Brian (La Jolla Cancer Research Foundation, La Jolla, CA). pGAPDH contains the full-length murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and was provided by Dr. M. Prystowsky (Albert Einstein School of Medicine, Bronx, NY). The plasmid containing the murine *c-myc* cDNA was obtained from American Type Culture Collection. The empty expression vector pBX and pBXEGR1 containing the full-length EGR1 cDNA driven by the SV40 promoter have been described (20). pSV₂PAP, containing the gene for placental alkaline phosphatase (PAP), was kindly provided by Dr. T. Kadesch (University of Pennsylvania).

pBLICAM and pBLmICAM contain 1.1 kb of the murine ICAM-1 5' flanking region including its transcription start site. A 1.1-kb BsiHKAI fragment spanning -1091 to +34 was excised from E10 (31), blunt ended with T4 polymerase, and cloned into the PstI site of the promoterless chloramphenicol acetyl transferase (CAT) vector pBLCAT2.3 (32). For construction of pBLmICAM, splicing overlap PCR (33) was used to mutate the EGR1-binding site located at -701 bp. The oligonucleotides used were 5'-GAGGCATATGGGCGGGAGC-3' and 5'-CCGCCCATATGCCTCGGTTCC-3'. Presence of the mutation was confirmed by sequencing using a Sequenase 2.0 kit (USB, Cleveland, OH) according to the manufacturer's instructions.

RNA Isolation and Northern Blot Analysis. Cells were brought to a concentration of 5×10^5 /ml in fresh 37°C media and then equilibrated for 1–2 h at 37°C, 5% CO₂. After preincubation, 10 ng/ml PMA, 10 μ g/ml goat anti-mouse μ F(ab')₂ (Chemicon International Inc., Temecula, CA), or 50 μ g/ml rabbit anti-mouse IgM F(ab')₂ was added as indicated in the text, and total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method (34). Equal amounts of RNA were electrophoresed on a 1.2% formaldehyde-agarose gel and blotted to nylon membranes (GeneScreen Plus; DuPont/NEN, Boston, MA), fixed by UV irradiation, and baked for 2 h at 80°C in a vacuum oven. cDNA probes were labeled using incorporation of [³²P]dCTP by nick translation (GIBCO BRL) according to the manufacturers instructions. After a 1–6 h prehybridization in 50 mM Pipes (pH 6.8), 50 mM NaPO₄, 100 mM NaCl, 1 mM EDTA, and 5% SDS at 65°C, denatured probe was added, and hybridization was carried out for 12–16 h at 65°C. Blots were washed at 65°C for 3 \times 20 min with 1 \times SSC and 5% SDS, 3 \times 20 min with 0.5 \times SSC and 0.1% SDS, and 3 \times 20 min with 0.2 \times SSC and 0.1% SDS. Quantitative analysis was carried out using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Nuclear Run-On Transcription Assays. Exponentially growing cells were aliquotted into 50-ml conical tubes and equilibrated at 37°C, 5% CO₂ for 2 h and stimulated with 10 ng/ml PMA. At the indicated times, cells were pelleted, washed twice with PBS at

4°C, and lysed in 1 ml of lysis buffer (10 mM Hepes, pH 8.5, 1.5 mM MgCl₂, 1.0 mM dithiothreitol, 2 mM PMSF, and 1.5 µg/ml each of pepstatin A, chymotrypsin, leupeptin, and antipain) on ice for 15 min. After one wash in lysis buffer, nuclei were resuspended in 1 ml of lysis buffer with 10 µg/ml RNase A for 30 min at 4°C to remove cytoplasmic RNA. Nuclei were then washed twice in lysis buffer at 4°C and resuspended at 10⁷ nuclei/100 µL in NSB (50% glycerol, 0.02 M Tris-Cl, pH 7.9, 0.075 M NaCl, 0.5 mM EDTA, 0.85 mM dithiothreitol, 0.125 mM PMSF), placed into a dry ice/EtOH bath for 20 min and stored at -70°C.

Run-on transcription was carried out at 26°C for 10 min in a 200-µL volume with reaction buffer (29% glycerol, 100 mM Tris-Cl, pH 7.9, 50 mM NaCl, 4.0 mM MnCl₂, 1.2 mM dithiothreitol, 0.1 mM PMSF, 0.4 mM EDTA, 1.0 mM nucleoside triphosphates, 10 mM phosphocreatine, 40 U RNAsin (Promega Corp., Madison, WI), 2.5 µM UTP, and 200 µCi [³²P]UTP) followed by addition of 1.5 U RNase-free DNase (Boehringer Mannheim Corp., Indianapolis, IN) and incubation at 26°C for an additional 5 min. Reactions were terminated by addition of 1 ml of guanidinium thiocyanate-phenol-NaOAc, pH 4.0, and RNA was extracted as described (35). Unincorporated nucleotides were removed by two successive NH₄OAc (2.5 M)/EtOH precipitations. Prehybridized Genescreen Plus membranes (DuPont/NEN, Boston, MA) containing 5 µg of denatured pBlue-script SK⁺ with or without inserts containing murine ICAM1 (pBlueICAM) or murine GAPDH (pGAPDH) were hybridized for 65 h at 42°C with 1.2 × 10⁷ cpm of nuclear run-on products in 1 ml of 50% formamide, 5× SSC, 50 mM NaPO₄, pH 7.0, 0.1% NaPP_i, 1% SDS, 1 mg/ml heparin, and 100 µg/ml sheared herring sperm DNA. Membranes were washed for 15 min at 22°C (2× SSC, 0.1% SDS) and 15 min at 68°C (0.1× SSC, 1% SDS). Quantitative analysis was performed as described above.

Electrophoretic Mobility Shift Assays. Exponentially growing cells were stimulated with 10 ng/ml PMA for 2 h, washed twice in PBS at 4°C, and nuclear extracts were prepared as described elsewhere (36, 37). The probe containing the BsaHI/DdeI fragment spanning -657 through -747 bp was labeled using T4 polynucleotide kinase according to the manufacturer's instructions (New England Biolabs, Inc., Beverly, MA) and purified on a 9% native polyacrylamide gel. Binding reactions were carried out in a 20-µl volume containing 10,000 dpm labeled probe, nuclear extract containing 10 µg of protein, 1 µg poly (dI-dC)-poly(dI-dC) (Pharmacia Biotech Inc., Piscataway, NJ), 10 mM Tris, pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM 2-ME and 4% glycerol with or without competing oligonucleotides as indicated in the text. For antibody-blocking experiments, antisera specific for either EGR1 or CDK4 (Santa Cruz Biotechnology, Santa Cruz, CA) were preincubated with nuclear extract and poly (dI-dC)-poly(dI-dC) for 15 min at room temperature. After addition of reaction buffer and labeled probe, the reaction was allowed to proceed for an additional 15 min at room temperature. Reactions were loaded onto a 4% polyacrylamide gel in 0.5× TBE (1× TBE is 100 mM Tris base, 89 mM boric acid, 2 mM EDTA) and electrophoresed at 120 V at room temperature.

Double-stranded oligonucleotides containing consensus binding sites for AP1 (5' CGCTTGATGAGTCAGCCGGA 3') and SP1 (5' ATTCGATCGGGGCGGGGCGAGC 3') (Promega Corp.), as well as consensus EGR1 (5' GGATCCAGCGGGGCGGAGCGGGGCGGA 3') and mutant EGR1 (5' GGATCCAGC-TAGGGCGAGCTAGGGCGGA 3'), the latter of which fails to bind to murine and human EGR1, were purchased from Santa Cruz Biotechnology.

Transient Transfections, CAT Assays, and PAP Assays. For tran-

sient transfections of WEHI-231, cells were grown to 4–5 × 10⁵ cells/ml in media supplemented with 100 U/ml penicillin and 100 µg streptomycin (supplemented WEHI-231 media). 10⁷ cells were washed once in STBS (25 mM Tris-Cl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na₂HPO₄, 0.7 mM CaCl₂, and 0.5 mM MgCl₂) and resuspended in 1.5 ml of 1× STBS, 0.5 mg/ml DEAE-dextran (Pharmacia Biotech Inc.), and the indicated plasmid DNA. After a 30 min incubation at room temperature, cells were washed once in STBS and resuspended at 2.5 × 10⁵ cells/ml in fresh supplemented WEHI-231 media. Cultures were divided and stimulated 28 h after transfection. After a 14-h stimulation, cells were harvested and assayed for CAT as described (32). To control for transfection efficiency, cells were cotransfected with pSV₂APAP expressing alkaline phosphatase, and enzymatic activity was determined as described (38).

Primary lymphocytes were transfected as described (29). Briefly, 2.5 × 10⁷ LPS-blasted cells were washed once in STBS and resuspended at 10⁷ cells/ml in STBS containing 500 µg/ml DEAE-dextran and the indicated plasmid DNA for 30 min at 37°C. Cells were then washed once in STBS and resuspended at 5 × 10⁶ cells/ml in fresh B cell assay media and incubated at 37°C, 5% CO₂. After 24 h, cells were split into two equal groups and either left unstimulated or stimulated with 10 ng/ml of PMA. After an additional 24-h incubation, cells were harvested and assayed for CAT.

Results

Differential Expression of EGR1 in Subclones of WEHI-231. We have previously reported a clone of the WEHI-231 B cell line (WEHI-231.7) that does not express *egr-1* upon anti-µ or phorbol ester stimulation (35). Lack of inducible expression of *egr-1* was shown to be due to gene-specific DNA methylation in these cells (39). Immunocytochemical analysis of the parental WEHI-231 cell line from which WEHI-231.7 was cloned demonstrated cell-to-cell heterogeneity with respect to *egr-1* expression (Monroe, J.G., unpublished observations). Therefore, a subsequent WEHI-231 clone was derived (WEHI-231.1F1) in which *egr-1* was expressed at both the message and protein levels after anti-µ or phorbol ester stimulation.

As shown in Fig. 1, cross-linking of the BCR with anti-µ antibodies or stimulation with the phorbol ester PMA resulted in a rapid induction of *egr-1* mRNA in WEHI-231.1F1 cells. In contrast, neither stimulus was able to induce detectable *egr-1* mRNA in WEHI-231.7 cells. Differential expression cannot be attributed to different kinetics of induction because no detectable *egr-1* mRNA in the WEHI-231.7 cells is observed at any time between 0.5 and 12 h of stimulation with anti-µ or PMA (35, 39). It is important to note here that as far as we have been able to ascertain, the observed difference in BCR- and PMA-induced *egr-1* in these lines relates to gene-specific transcriptional silencing (39) and is not associated with quantitative differences in BCR expression or signaling. Both lines exhibit comparable sIgM expression levels (Fig. 2 A) and undergo anti-µ- and PMA-induced growth arrest (Maltzman, J.S., and J.G. Monroe, unpublished data). Furthermore, the difference seen in *egr-1* message is not representative of a generalized

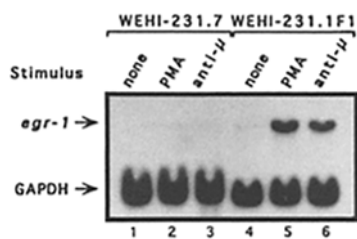


Figure 1. WEHI-231 differ in their anti- μ - and PMA-induced expression of *egr-1*. WEHI-231.7 or WEHI-231.1F1 were either left unstimulated (lanes 1 and 4) or were stimulated for 1 h with either 10 ng/ml PMA or 50 μ g/ml rabbit anti-mouse μ F(ab')₂. Total cellular RNA was size fractionated by electrophoresis through 1.2% agarose-formaldehyde gel, transferred to GeneScreen Plus membranes, and hybridized simultaneously with ³²P-labeled probes for *egr-1* and GAPDH as described in Materials and Methods.

inability to induce immediate-early gene expression, since the expression of other transcription factors encoded by immediate-early genes such as *c-myc* (Fig. 2 B) and *egr-2/krox-20* (data not shown) does not differ between these two lines. Thus, although we are unable to formally exclude the possibility that WEHI-231.7 and WEHI-231.1F17 (hereafter referred to as 231.7 and 1F1, respectively) may differ in parameters other than *egr-1* inducibility, this pair of related and otherwise phenotypically similar cells affords us the opportunity to identify genes whose transcription is regulated directly or indirectly by EGR1. We reasoned that BCR-induced genes that require EGR1 expression for

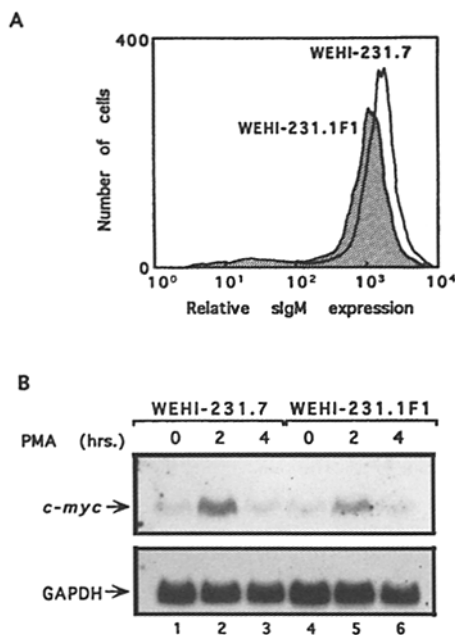


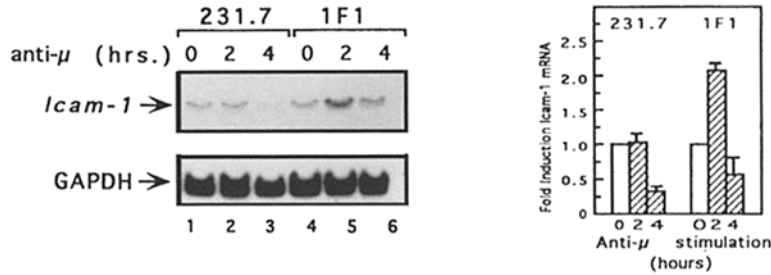
Figure 2. Relative expression of surface IgM and inducible *c-myc* in WEHI-231.7 and WEHI-231.1F1. (A) Surface IgM levels were compared by flow cytometric analysis of WEHI-231.7 (open area) and WEHI-231.1F1 (shaded area) for relative levels of surface IgM expression. (B) The inducible expression of *c-myc* was analyzed by Northern blot analysis. WEHI-231.7 or WEHI-231.1F1 were either left unstimulated or were stimulated for the indicated times with 10 ng/ml PMA. Total cellular RNA was subjected to Northern blot analysis and probed sequentially with ³²P-labeled cDNA probes for *c-myc* (top) and GAPDH (bottom).

their induction would show similar differential induction patterns in 1F1 and 231.7 B cells.

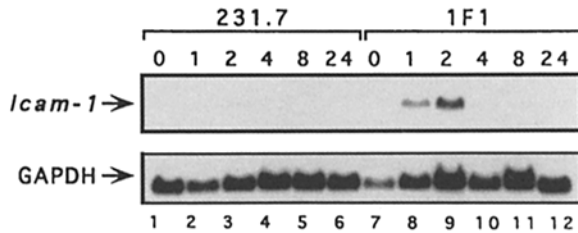
Induction of *Icam-1* Gene Expression is Associated with EGR1 Expression in 1F1 and 231.7 Cells. For the reasons discussed previously, we considered *Icam-1* to be a potential target of regulation by EGR1. Consistent with this possibility, anti- μ stimulation of the EGR1-nonexpressing line (231.7) failed to induce increased *Icam-1* mRNA expression (Fig. 3 A). Failure to induce expression of *Icam-1* mRNA contrasted with the response observed in the EGR1-expressing subclone 1F1, in which we observed induction of *Icam-1* mRNA by 2 h after stimulation with anti- μ . Induction by anti- μ was small (\sim 2-fold at 2 h), but this induction was significantly different ($P < 0.01$) than that seen in 231.7 cells across four independent experiments (Fig. 3 A, right). Bypassing the BCR by stimulating the cells with PMA also resulted in *Icam-1* induction in 1F1 but not the *egr-1*-nonexpressing line 231.7 (Fig. 3 B). The results of this latter experiment were quantitated and are presented in Fig. 3 C. As with anti- μ stimulation, significant differences in *Icam-1* inducibility were observed in 231.7 and 1F1 cells; in this case, PMA induced a 16-fold increase in *Icam-1* mRNA expression. Differential induction between 231.7 and 1F1 was consistent over five independent experiments. Greater induction of *Icam-1* mRNA by PMA than by anti- μ stimulation is consistent with our previous observations that PMA is more efficient than anti- μ in its ability to induce *egr-1* expression (19; see Figs. 1 and 6).

Differential Induction of *Icam-1* Is Due to a Difference in the Rate of Transcription Initiation. Regulation of *Icam-1* transcription by EGR1 would predict that, minimally, a component of the difference seen in the induced steady state *Icam-1* mRNA levels would be due to differences in the rate of transcriptional initiation of the *Icam-1* gene. To determine differential induction of *Icam-1* transcription in stimulated 231.7 and 1F1 B cells, we carried out nuclear run-on analyses. Nuclei from 231.7 and 1F1 cells were isolated at various times after stimulation with PMA. The results for 1F1 cells are shown in Fig. 4 A. The rate of transcription initiation of *Icam-1* was increased at 1 h (relative to unstimulated controls) after stimulation of 1F1 cells. The rate of transcription began to decrease by 2 h and was down to basal levels by the 3-h time point, consistent with the kinetics observed by Northern blot analysis (Fig. 3). To quantitatively compare the induction of *Icam-1* transcription between 1F1 and 231.7, identical analyses were performed using 231.7 nuclei, and band intensities were quantified and normalized to levels of transcription of the housekeeping gene encoding GAPDH. The results of these analyses are expressed graphically in Fig. 4 B. As indicated, stimulation of 1F1 cells led to a sevenfold increase in transcription at 1 h compared with a threefold increase in 231.7 cells. These results indicate that the differential levels of induction seen at the steady state level by Northern blot analysis are due at least in part to differences in the rate of *Icam-1* transcription, consistent with EGR1-dependent regulation. It is not obvious how differences in the level of inducibility as measured by Northern blot analysis (Fig. 3 B;

A



B



C

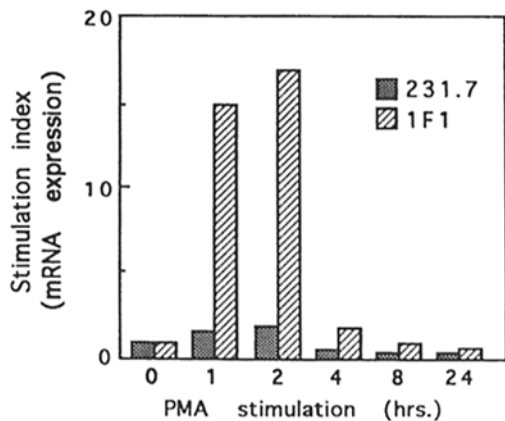


Figure 3. Comparison of *Icam-1* mRNA levels in anti- μ - and PMA-stimulated 231.7 and 1F1 cells. 231.7 and 1F1 cells were either left unstimulated or were stimulated with (A) 10 μ g/ml of F(ab')₂ fragments of goat anti-mouse μ antibodies (left and right) or (B) 10 ng/ml PMA for the times indicated. Total RNA was size fractionated on 1.2% agarose-formaldehyde gels, transferred to GeneScreen Plus membranes, and sequentially hybridized with ³²P labeled cDNA probes for *Icam-1* (A and B, top) and GAPDH (A and B, bottom). The right panel of A depicts the means of four independent experiments presented as the mean fold induction relative to unstimulated cells \pm SEM (*Icam-1* mRNA expression levels were first normalized to GAPDH levels). (C) Quantitative analysis of PMA-stimulated *Icam-1* and GAPDH RNA levels from B was performed using a PhosphorImager as described in Materials and Methods. For each time point, the level of *Icam-1* was normalized to the level of GAPDH. Fold induction is the normalized level of *Icam-1* message at the time indicated divided by the normalized level of unstimulated *Icam-1*.

16-fold) and transcription initiation are to be reconciled. Often, the levels measured by these two assays are not identical; run-ons routinely show less induction than Northern blot analysis. This difference may reflect characteristics of the assay systems themselves, that is, efficiency and sensitivity or, in some cases, suggest a degree of post-transcriptional regulation in the induced cells. The critical point in this analysis, however, is the clear evidence of transcriptional induction of *Icam-1* in 1F1 and the elevated induction in 1F1 cells compared with that observed in 231.7 B cells.

EGR1 Binds to a Consensus Motif at -701 bp of the Murine Icam-1 Promoter. Sequence analysis of the human *Icam-1* promoter suggested two potential EGR1-binding motifs at -693 bp and -699 bp. At the onset of these studies, the sequence of the murine promoter had not been extended

beyond -660 bp (31), and therefore could not be evaluated for the presence of these potential regulatory motifs. Further sequencing of the murine 5' region and analysis of the region between -660 and -1091 bp identified a single consensus EGR1-binding motif at -701 bp, an AP2 motif at -883 bp, an NF- κ B motif at -870 bp, and two SP1-binding motifs at -700 and -955 bp (shown schematically in Fig. 5 A).

To determine if murine EGR1 protein binds to the motif at -701 bp, nuclear extracts isolated from stimulated 1F1 cells were used as a source of cellular EGR1 protein in electrophoretic mobility shift assays (EMSA). Competition EMSA analysis was carried out using a radiolabeled 90-bp probe spanning the region from -747 to -657 bp (Fig. 5 A). Addition of nuclear extracts from stimulated 1F1 cells resulted in multiple shifted complexes (Fig. 5 B, lane 1).

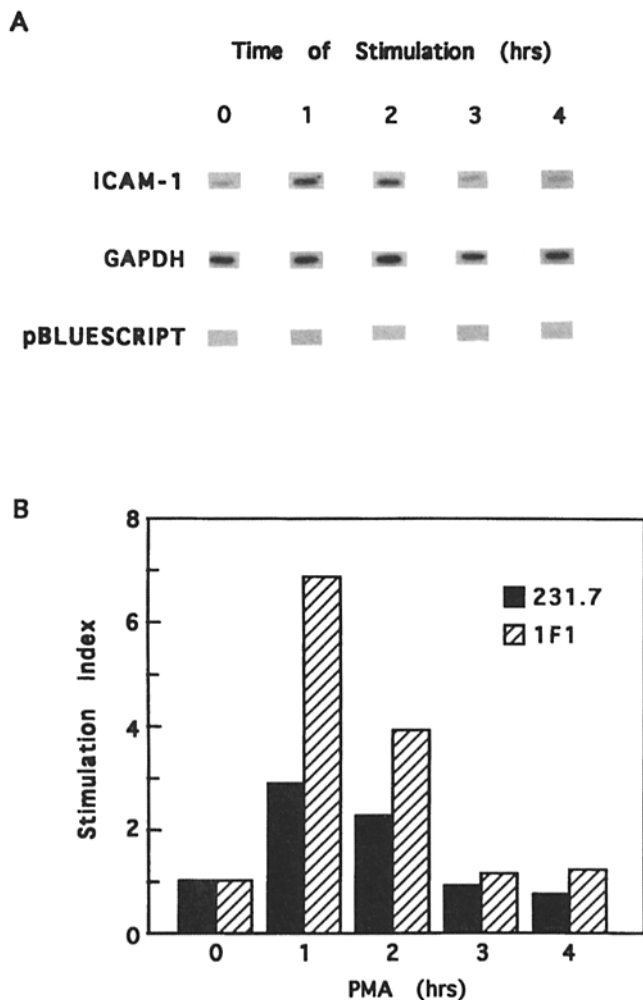


Figure 4. Differential induction of *Icam-1* transcription initiation in 231.7 and 1F1 cells. (A) Nuclei were isolated from 1F1 cells that had been stimulated with 10 ng/ml PMA for 0, 1, 2, 3, or 4 h and used as templates to produce ^{32}P labeled nuclear run-on transcription probes as described in Materials and Methods. 5 μg each of pBluescript SK⁺ vectors containing either *Icam-1*, GAPDH, or no insert were immobilized onto GeneScreen Plus membranes. The run-on transcription products were hybridized to the membranes. (B) Quantitative analysis of PMA-stimulated *Icam-1* transcription in 231.7 and 1F1 cells was performed using a PhosphorImager. *Icam-1* band intensities from the hybridized filters from A and a simultaneous experiment using nuclei from PMA-stimulated 231.7 cells were quantitated and normalized to GAPDH intensities at each time point. The results are expressed as fold induction versus unstimulated levels.

Competition with oligonucleotides containing two consensus EGR1-binding motifs (*ERE*), but not mutated EGR1 binding motifs (*mERE*), resulted in dose-dependent competition of a single intermediate-sized complex (Fig. 5 B, compare lanes 2 and 3 with 1 and 4).

The potential EGR1-binding site at -701 bp is associated with an overlapping SP1 site. Therefore, the slower mobility complex present in the lanes containing extracts from either 231.7 or 1F1 cells was considered to be SP1. This conclusion was confirmed by experiments shown in Fig. 5 B in which oligonucleotides containing a consensus

SP1-binding motif specifically competed for the slower complex mobility (Fig. 5 B, compare lanes 1 and 5). Conversely, oligonucleotides containing an AP1-binding motif did not compete for binding of any of these complexes (Fig. 5 B, compare lanes 1 and 6), confirming the sequence specificity of these complexes.

To confirm that the complex competed by unlabeled EGR1 oligonucleotides contained EGR1 and not a related family member, two types of experiments were performed. First, addition of rabbit antiserum specific for a COOH-terminal region of the EGR1 that is not shared by other EGR family members (40–43) resulted in a specific supershift of the intermediate mobility complex that was previously shown to be disrupted by competition with unlabeled EGR1- but not SP1-binding sites (Fig. 5 C). In contrast, a control rabbit antiserum did not supershift or disrupt the complex. In a second type of experiment, nuclear extracts from stimulated and unstimulated 231.7 and 1F1 cells were compared (Fig. 5 D). If the protein participating in formation of the intermediate mobility complex is EGR1, then this complex would be expected to be unique to 1F1 nuclei (compared with 231.7) and also should be more abundant in stimulated extracts. In comparison, since SP1 is a ubiquitously expressed transcription factor (44), its binding activity should be observed in both subclones. As shown in Fig. 5 D, the EGR1 complex is unique to the 1F1 cells, and its abundance increases with stimulation. In addition, the slow mobility complex identified as SP1 by competition experiments is present in both cell lines regardless of stimulation (Fig. 5 D). In contrast to EGR1, increased binding of SP1 after stimulation of 1F1 cells is not a reproducible finding in our hands.

Involvement of the -701-bp EGR1-binding Site in *Icam-1* Induction. The above studies have identified a potential EGR1-binding site in the 5' *Icam-1* promoter and demonstrated the ability of this site to bind to EGR1. To determine whether this site is important for the transcriptional regulation of *Icam-1* in anti- μ - and PMA-stimulated B lymphocytes, we mutated this site in the context of the entire *Icam-1* promoter. Reporters containing either 1.1 kb of the murine *Icam-1* promoter (-1091 to +34 bp) upstream of the CAT reporter gene (pBLICAM), or a variant derived by site-directed mutagenesis of the EGR1-binding site at -701 bp (pBLmICAM) were constructed (see Fig. 7 A). The mutation within the pBLmICAM construct consists of a 5-bp substitution that abolishes the ability of EGR1 to bind to the -701-bp site but, importantly, retains SP1-binding activity as determined by EMSA (data not shown). To test the effect of this mutation on the ability to induce *Icam-1* gene expression in anti- μ - and PMA-stimulated B cells, we exploited the use of the LPS blast transfection system that we have previously described (29). The strength of this is that it allows us to analyze inducibility in a non-transformed primary B cell; caveats associated with transformation of established tissue culture lines are thus avoided. Importantly, both *Icam-1* and *egr-1* are inducible by PMA and anti- μ in the LPS blast system (Fig. 6).

B lymphocytes, transiently transfected with either pBL-

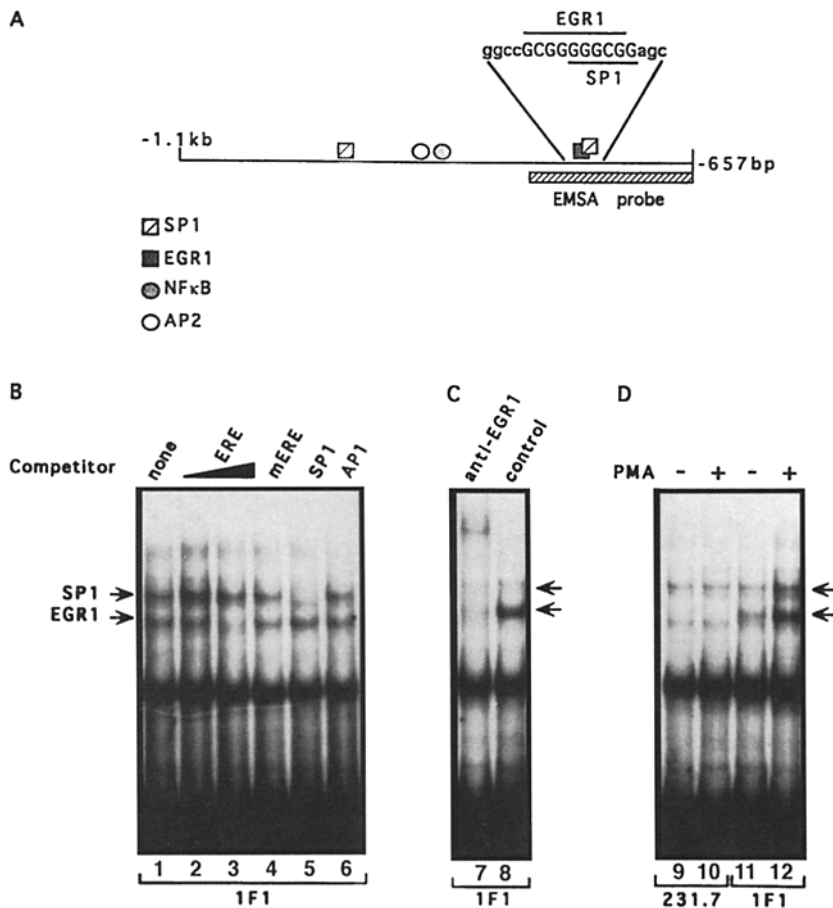


Figure 5. EGR1 binds to a region located at -701 bp of the murine *Icam-1* promoter. (A) Schematic representation of the distal murine *Icam-1* promoter indicating the radiolabeled probe used for EMSA (hatched bar). The probe spans from -657 to -747 bp. The basepair sequence from -697 to -713 bp is shown to indicate the overlapping EGR1 and Sp1 consensus-binding motifs and the immediately flanking sequences. Also shown schematically are potential binding motifs for NF- κ B (-870 bp), AP2 (-883 bp), and an additional SP1 motif (-955 bp). Gel mobility shift assays were performed using $5 \mu\text{g}$ of nuclear extracts from 1F1 cells stimulated for 2 h with 10 ng/ml PMA and the ^{32}P labeled probe depicted in A. To identify the proteins in DNA-protein complexes, competing oligonucleotides (B) or antisera (C) were preincubated with extract for 10 min at 4°C , the probe was added, and binding was allowed to proceed for an additional 15 min at room temperature. Unlabeled oligonucleotide competitors were used at either 20-fold molar excess (EGR1 response element [ERE], low concentration) or 100-fold molar excess (remaining competitors). The supershifted complex in C with the anti-EGR1 antibody is indicated by the (*). (D) Nuclear extracts from either 231.7 or 1F1 that were unstimulated or stimulated with 10 ng/ml of PMA for 2 h were incubated with the probe as described in B.

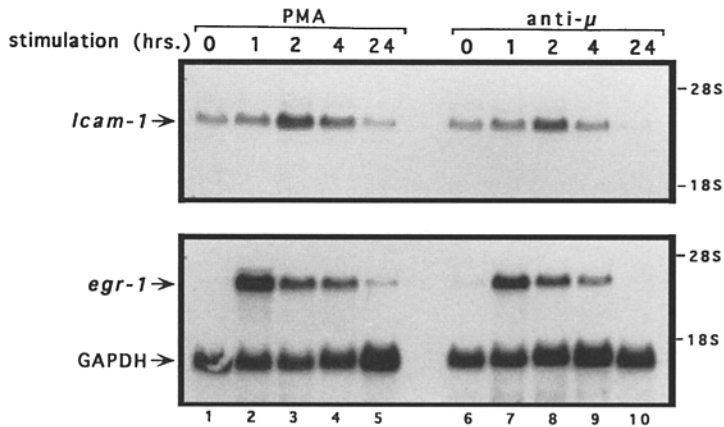
ICAM or pBLmICAM, were left unstimulated or were stimulated with anti- μ (Table 1) or PMA (Fig. 7 and Table 1). The level of basal CAT activity from unstimulated cells was approximately equal whether the EGR1 motif was intact or mutated (Fig. 7 B). Marked induction (~ 25 -fold) of *Icam-1* promoter activity was observed after PMA stimulation of cells transfected with the wild-type construct. Identical stimulation of the cells transfected with the mutated promoter resulted in a 50–75% reduction in inducible promoter activity relative to induction of the wild-type construct. Abrogation of transcription induction of the *Icam-1* promoter by mutation of the -701 -bp EGR1-binding site was observed in five separate experiments in which PMA was the stimulus (Table 1). Importantly, although induction by anti- μ stimulation is less than that observed for PMA, mutation of the defined EGR1-binding site nonetheless abrogated induced levels of transcriptional activity by 48–68% across three separate experiments (Table 1). From these studies, we can conclude that the -701 -bp EGR1-binding motif is required for the full induction of the *Icam-1* promoter.

Exogenous EGR1 Transactivates the *Icam-1* Promoter. The above results show that the EGR1 motif (independent of the overlapping SP1 motif) is necessary for full activation of the *Icam-1* promoter in anti- μ - or PMA-stimulated B cells.

Coupled with the studies using the 231.7 and 1F1 B cell lines, these data provide strong correlative evidence for the importance of EGR1 in BCR-inducible expression of *Icam-1*. To more directly determine a role for the EGR1 protein in *Icam-1* gene regulation, we tested the ability of plasmid expressed EGR1 to transactivate the *Icam-1* promoter. For these studies, we used the 231.7 cells so that the experiments would not be complicated by endogenous EGR1 expression. 231.7 B cells were cotransfected with the wild-type *Icam-1* promoter/CAT reporter gene (pBL-ICAM) and varying amounts of an SV40-driven murine EGR1 expression vector (pBXEGR1). Transfections were performed in PMA-stimulated 231.7 B cells, maintaining equivalent amounts of expression vector DNA ($5 \mu\text{g}$) by inclusion of appropriate amounts of the empty cassette, pBX. As can be seen (Fig. 8 B, solid bars), we observed a dose-dependent increase in *Icam-1* promoter activity associated with increasing amounts of EGR1 expression vector.

In the above case, EGR1 could be acting directly or indirectly through this promoter. However, we believe that this effect is mediated by direct effects of EGR1 on the *Icam-1* promoter because mutation of the -701 -bp EGR1-binding site completely abrogates the EGR1-mediated transactivation (Fig. 8 B, hatched bars). Therefore, the transactivation studies clearly establish the ability of EGR1 to

A



B

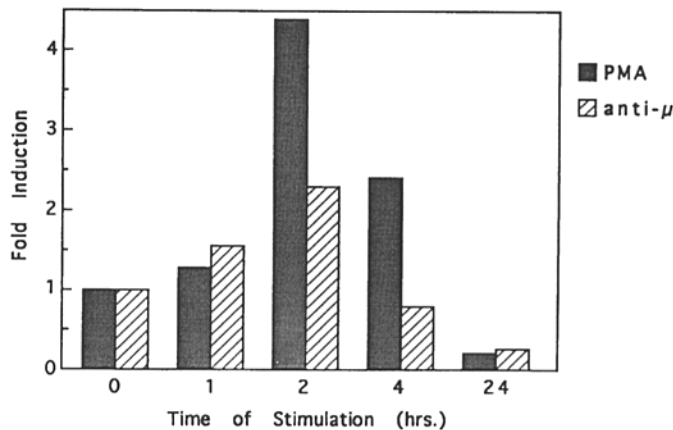


Figure 6. BCR cross-linking and PMA stimulation induce *Icam-1* and *egr-1* mRNA in LPS blasts. Purified splenic B lymphocytes were stimulated for 72 h with 50 $\mu\text{g}/\text{ml}$ of LPS. Cells were washed twice with PBS and cultured for an additional 24 h in the absence of LPS. Cells were then stimulated with either 10 ng/ml PMA (lanes 1–5) or 10 $\mu\text{g}/\text{ml}$ of F(ab')₂ fragments of goat anti-mouse μ (lanes 6–10) for the times indicated above each lane. Equivalent amounts of RNA were size fractionated, transferred to a GeneScreen Plus membrane, and hybridized with ³²P labeled cDNA probes for *Icam-1* (A, top). The membrane was stripped and simultaneously reprobed for *egr-1* and GAPDH (A, bottom). The relative locations of 28S and 18S rRNA are indicated to the right. (B) The levels of *Icam-1* mRNA expression were quantitated and normalized to GAPDH expression. Values are presented as fold induction after PMA or anti- μ stimulation relative to unstimulated cells.

induce transcription via the *Icam-1* promoter and, furthermore, establish that this effect is most likely to be direct. The observation that mutation of the *Icam-1*-associated EGR1-binding site abolishes EGR1 transactivation of this promoter indicates that EGR1 is not acting indirectly via regulation of another transcription factor.

Discussion

Activation of B lymphocytes by antigen is a complex process that integrates membrane, cytoplasmic, and nuclear processes into long-term phenotypic and functional changes in the B cell. These activation-associated events regulate the ability of the B cell to present processed antigen to T cells, function as an immune effector cell, and generate antigen-specific memory cells. Transcription factors whose expression is induced after BCR signaling facilitate coupling between receptor-induced second-messenger pathways and the nuclear events that regulate these phenotypic and functional changes. Here we have investigated a single and defined event that is necessary for an antigen-induced immune response (45, 46). We have shown that induction of *Icam-1* transcription after BCR-induced signals is coupled to the BCR via the transcription factor EGR1. In our

previous studies, we identified the p21^{ras/raf}/MAP kinase pathway as the important signaling pathway linking BCR or phorbol ester stimulation to *egr-1* gene induction (47). Taken together, these studies define a role for the p21^{ras} pathway and EGR1 in linking BCR cross-linking to immunologically relevant functional responses in B cells; namely, the upregulation of expression of a gene directly involved in B cell-T cell interaction. Adhesion molecules such as ICAM-1 function to stabilize interactions with T cells during the period in which the B cells manifest a requirement for secondary signals to further promote activation and differentiation of the antigen-stimulated B cell.

The importance of EGR1 in the induction of *Icam-1* expression after BCR cross-linking is based on several lines of mutually supporting evidence: (a) we have demonstrated that the -701-bp EGR1-binding site is necessary for the full inducible activity of the *Icam-1* gene in response to either BCR ligation or in situations in which the receptor is bypassed by stimulation with phorbol ester; (b) B cell lines that differ in their ability to express EGR1 also show differential inducibility of *Icam-1* gene expression; and (c) exogenous EGR1 is able to transactivate the *Icam-1* promoter, and mutation of the -701-bp EGR1-binding site abolishes this transcriptional activation. The caveats and interpreta-

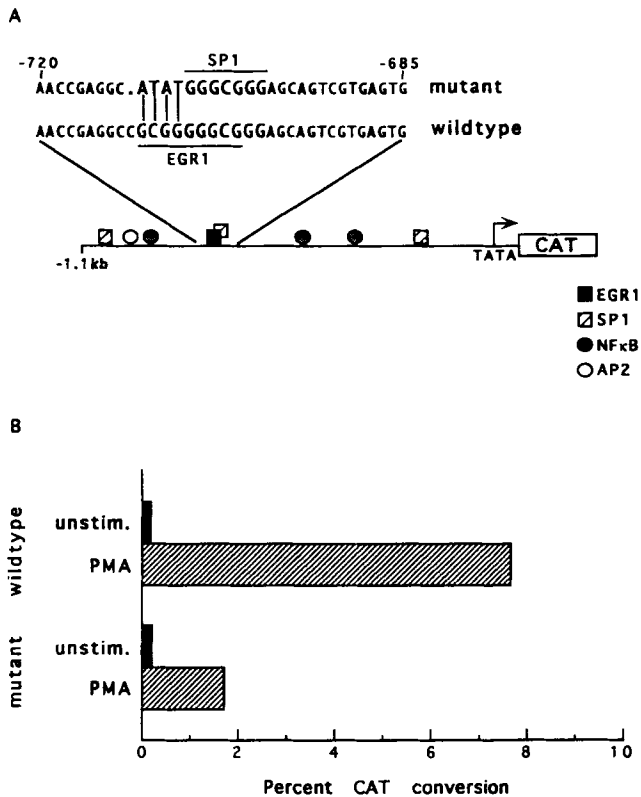


Figure 7. Mutation of the EGR1-binding site at -701 bp abrogates inducible *Icam-1* promoter activity. (A) pBLICAM contains 1.1 kb of 5' flanking sequence from the murine *Icam-1* gene cloned upstream of a CAT reporter. The mutated construct (pBLmICAM) contains the depicted 5-bp mutation. (B) After a 72-h pretreatment with LPS, B cells were transfected with 10 μ g of either pBLICAM (wild type) or pBLmICAM (mutant) and rested for 24 h. Cultures were then evenly divided, and cells were either stimulated with 10 ng/ml PMA (hatched bars) or left unstimulated (solid bars). Cells were harvested 24 h later and assayed for CAT activity as described in Materials and Methods. Different preparations of LPS and each plasmid gave similar results.

Table 1. Both PMA- and Anti- μ -stimulated *Icam-1* Gene Transcription Is Abrogated by Mutation of the EGR1-binding Site at -701 bp

Fold induction reporter	Stimulus	Experiment				
		1	2	3	4	5
Wild-type <i>Icam-1</i>	PMA	28.1	25.5	26.9	21.2	20.0
Mutant (-701 bp)	PMA	9.1	10.3	10.9	9.4	11.4
Wild-type <i>Icam-1</i>	Anti- μ	1.7	2.5	5.1		
Mutant (-701 bp)	Anti- μ	0.7	0.8	2.7		

Experiments represent individual separate experiments using the wild-type and mutant *Icam-1* promoter-CAT reporter constructs described in Fig. 7. Primary B cells (LPS blasts) were transfected with pBLICAM (wild-type) or pBLmICAM (mutant -701 bp) and stimulated with either PMA (10 ng/ml) or anti- μ antibodies (50 μ g/ml) as described in Fig. 7. Results are fold induction relative to unstimulated control cultures.

tions of each of these conclusions will be discussed in detail below.

Whereas mutation of the -701 -bp EGR1-binding site abolishes as much as 75% of the inducible activity of the *Icam-1* gene in B cells, it does not abolish all inducible activity, suggesting regulation by other as yet unidentified transcription factors. Clearly, all *Icam-1* expression in B cells is not dependent upon EGR1 expression. In 231.7 B cells, which do not express EGR1, as well as resting 1F1 and primary B cells, significant levels of *Icam-1* mRNA expression are detectable despite the relative lack of measurable *egr-1* expression (see Figs. 3 and 6). We interpret these findings to indicate that whereas BCR-inducible *Icam-1* expression is to a large extent dependent upon EGR1, basal or resting levels are regulated by another mechanism. The SP1 site

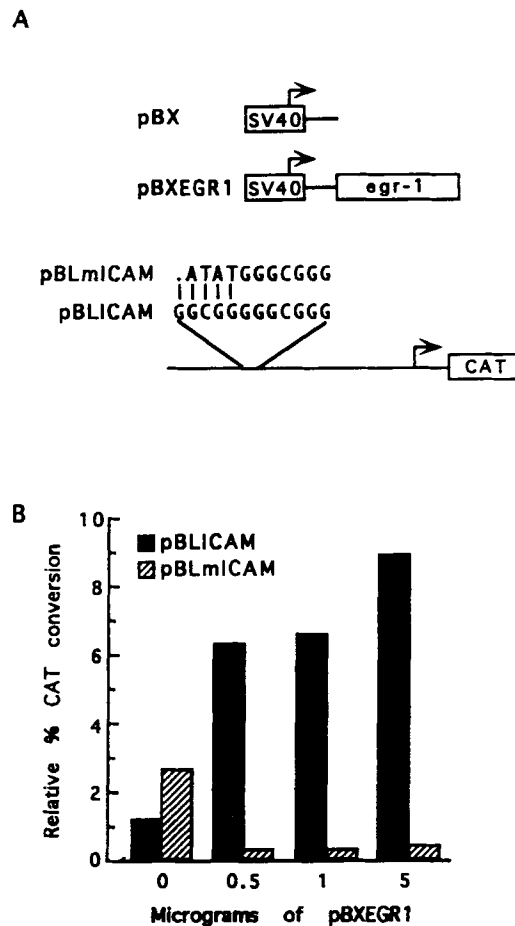


Figure 8. EGR1 is able to transactivate the *Icam-1* promoter through the -701 bp site. Transient cotransfections were carried out using 231.7 cells and 30 μ g of plasmid DNA containing a mixture of 20 μ g *Icam-1* reporter plasmid (pBLICAM or pBLmICAM), 5 μ g expression vector (combination of pBXEGR1 plus the empty expression vector pBX), and 5 μ g pSV2PAP to control for transfection efficiency. The plasmids used are illustrated in A. In B, the wild-type reporter construct pBLICAM (solid bars) or mutated pBLmICAM (hatched bars) were cotransfected with the indicated amount of pBXEGR1 and enough pBX to bring the total amount of expression vector DNA to 5 μ g. 32 h after transfection, cells were stimulated with 10 ng/ml of PMA. Cells were harvested after 14 h of stimulation, and CAT and PAP activities were quantitated as described in Materials and Methods. The relative CAT activity is shown.

may be important for this regulation. As discussed previously, SP1 is a constitutively expressed transcription factor in these cells, and, as shown in the EMSA experiments, it binds to the promoter in the unstimulated cells. Its role in the inducible expression of *Icam-1* was not directly evaluated in these studies because the integrity of the SP1-binding site was maintained in the mutations of the -701-bp site. The fact that inducible activity was reduced by 50–75% despite the ability of SP1 to still bind to this site indicates that it is not the major regulator of inducible *Icam-1* expression. However, it is still possible that the inducible activity remaining when EGR1 binding is abolished may be due to the intact binding of SP1 to this site. Changes in the levels of SP1 or its posttranslational modification in response to BCR or PMA stimulation may contribute to the activity of the *Icam-1* promoter under conditions in which EGR1 is absent or cannot bind. Alternatively, other sites in the promoter may also contribute to the inducible activity of this promoter after BCR or PMA stimulation. For example, an NF- κ B-binding motif has been implicated in cytokine-induced *Icam-1* expression in human endothelial cells (13, 48, 49). Regardless of the contributions of either of these mechanisms to the inducible activity of the promoter in the absence of EGR1 binding, our data clearly implicate EGR1 as the major regulator of *Icam-1* transcription in BCR- and PMA-stimulated B cells.

Before leaving this subject, it could be argued that it is a protein other than EGR1 that binds to the -701 motif and regulates the transcriptional activity of this promoter. Several lines of evidence argue against this possibility, however. First and foremost is the observation that induction of *Icam-1* is compromised in the 231.7 cells that fail to express EGR1 but do not differ in their expression of the other *egr* family member (*egr-2*), which can also bind this site (49a). Secondly, the EMSA analysis of the -701-bp region shown in Fig. 5 identified a single band that was associated with the ability to induce *Icam-1* expression (i.e., this band comigrated with a complex associated with the -701-bp site in EGR1-expressing but not nonexpressing B cells and was specifically blocked with a consensus EGR1-binding site but not a mutated one). Most importantly, the complex associated with this band was shown to be disrupted with antibodies specific to the EGR1 protein. Finally, the transactivation studies establish the ability of EGR1 to activate this promoter through this motif. In the 231.7 EGR1 nonexpressors, even after stimulation with PMA, significant induction required exogenous EGR1 expression. Taken together, these results indicate strong support for our interpretation that EGR1 is the relevant transcription factor in this response.

Because 231.7 B cells do not express EGR1, we were able to perform the transactivation studies using stimulated cells. By doing so, we were not only able to test the ability of EGR1 to transactivate this promoter, but also to establish the importance of EGR1 under conditions in which any other putative induced transcriptional regulators would be present. The observation that significant *Icam-1* promoter activity required the expression of EGR1 and that

this transactivation depended upon the ability of EGR1 to bind this promoter (i.e., mutation of the -701 site blocked this response) demonstrates three things. First, EGR1 can activate this promoter. Second, the lack of *Icam-1* inducibility in 231.7 is not due to a repressor of transcription, because this putative repressor would be expected to inhibit transactivation by the exogenous EGR1 protein. Third, significant promoter activity requires EGR1 even under conditions in which other potential transactivators may be present.

Interestingly, in light of this discussion, when similar studies were performed in unstimulated 231.7 cells, we failed to observe transactivation of the *Icam-1* promoter. We conclude, therefore, that stimulation is necessary for transactivation by exogenous EGR1 in this system. Although this result does not negate the conclusion relevant to the studies here (i.e., that EGR1 does transactivate this promoter), it does raise some interesting possibilities regarding the mechanism of EGR1 transcriptional regulation. First and less interesting, the requirement for stimulation may reflect the need to stimulate higher levels of transfected EGR1 expression. In this regard, it is well-established that stimulation can increase the activity of the SV40 enhancer. We do not believe that this is the case, however, because we observed similar effects using the β -actin promoter, which is not affected by PMA or anti- μ stimulation (data not shown). Also, as alluded to previously, stimulation may induce expression of other cofactors that cooperate with EGR1 in the transcriptional regulation of this promoter (50–52). Alternatively, EGR1 may require post-translational processing provided by stimulation, such as phosphorylation, to exert its activation effect (53–55). In this regard, it is interesting that the major transactivation domain of EGR1 is rich in extended stretches of serine and threonine residues (20). Phosphorylation of these residues would be expected to impart a net negative charge to this region, and in so doing, possibly convert it from an inactive to active transcriptional activator. EGR1 is phosphorylated on serine residues in fibroblasts stimulated with serum (56), and this phosphorylation is associated with increased EGR1 transcriptional activity (57, 58). Evaluating the relative contribution of these effects is a current area of interest in our laboratory.

Identification and use of the 231.7 and 1F1 B cells lines is a significant accomplishment of these studies. Their use in these studies afforded us two unique advantages over other systems used to evaluate transcription factor–target gene relationships. First (as just discussed), they allowed us the opportunity to carry out transient cotransfection assays in stimulated cells without the expression of endogenous EGR1. By comparing gene regulation in stimulated B cells with and without EGR1, we were able to evaluate the relative importance of EGR1 expression in the presence of other stimulation-associated processes. This level of analysis is not possible in the majority of model systems in which the stimulus would induce expression of the endogenous transcription factor as well as these other potential events. Perhaps even more important, comparisons between 231.7

and 1F1 have allowed us to evaluate *Icam-1* regulation by EGR1 at physiological levels of EGR1 (endogenous expression in 1F1) and with the *Icam-1* gene in its normal chromosomal context. Whereas transient assay systems as used in the majority of published studies allow for detailed deletion/mutation mapping of promoter elements, the DNA reporters used in these studies lack the constraints imposed by chromosome structure, which limits the interpretation of studies relying fully on this type of experiment.

The 231.7/1F1 system offers the opportunity to examine the involvement of EGR1 on regulation of endogenous genes. The combination of transient expression data and endogenous gene regulation makes this a unique system for studies of EGR1-regulated genes. Importantly, the cell line studies are further strengthened by the LPS blast system, which has allowed us to confirm and extend these studies using a nontransformed B cell model.

We would thank Dr. Marian Birkeland for critical reading of this manuscript. We thank C. Ballantyne for the murine *Icam-1* genomic clone used in the construction of CAT vectors.

This work was funded by National Institutes of Health (NIH) grants AI-23568 and AI-32592. J.S. Maltzman was partially funded by NIH Medical Scientist Training Program grant 5-T32-GM-07170.

Address correspondence to John G. Monroe, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Room 538A Clinical Research Building, 415 Curie Boulevard, Philadelphia, PA 19104.

Received for publication 31 August 1995 and in revised form 20 December 1995.

References

1. Cambier, J.C., C.M. Pleiman, and M.R. Clark. 1994. Signal transduction by the B cell antigen receptor and its coreceptors. *Annu. Rev. Immunol.* 12:457-486.
2. Parker, D.C. 1993. T cell-dependent B cell activation. *Annu. Rev. Immunol.* 11:331-360.
3. Scholl, P.R., and R.S. Geha. 1994. MHC class II signaling in B-cell activation. *Immunol. Today.* 15:418-422.
4. Durie, F.H., T.M. Foy, S.R. Masters, J.D. Laman, and R.J. Noelle. 1994. The role of CD40 in the regulation of humoral and cell-mediated immunity. *Immunol. Today.* 15:406-411.
5. Poudrier, J., and T. Owens. 1994. CD54/intercellular adhesion molecule 1 and major histocompatibility complex II signaling induces B cells to express interleukin 2 receptors and complements help provided through CD40 ligation. *J. Exp. Med.* 179:1417-1427.
6. Lane, P.J.L., F.M. McConnell, E.A. Clark, and E. Mellins. 1991. Rapid signaling to B cells by antigen-specific T cells requires CD18/CD54 interaction. *J. Immunol.* 147:4103-4108.
7. de Fougères, A.R., and T.A. Springer. 1992. Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. *J. Exp. Med.* 175:185-190.
8. Staunton, D.E., M.L. Dustin, and T.A. Springer. 1989. Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. *Nature (Lond.)*. 339:61-64.
9. Dustin, M.L., and T.A. Springer. 1991. Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. *Annu. Rev. Immunol.* 9:27-66.
10. Deleted in proof.
11. Dang, L.H., and K.L. Rock. 1991. Stimulation of B lymphocytes through surface Ig receptors induces LFA-1 and ICAM-1 dependent adhesion. *J. Immunol.* 146:3273-3279.
12. Branden, H., and E. Lundgren. 1993. Differential regulation of LFA-1 and ICAM-1 on human primary B-lymphocytes. *Cell. Immunol.* 147:64-72.
13. Voraberger, G., R. Schafer, and C. Stratowa. 1991. Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5'-regulatory region. Induction by cytokines and phorbol ester. *J. Immunol.* 147:2777-2786.
14. Wertheimer, S.J., C.L. Myers, R.W. Wallace, and T.P. Parks. 1992. Intercellular adhesion molecule-1 gene expression in human endothelial cells: differential regulation by tumor necrosis factor- α and phorbol myristate acetate. *J. Biol. Chem.* 267:12030-12035.
15. Milbrandt, J. 1987. A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science (Wash. DC)*. 238:797-799.
16. Lemaire, P., O. Revelant, R. Bravo, and P. Charnay. 1988. Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. *Proc. Natl. Acad. Sci. USA.* 85:4691-4695.
17. Christy, B.A., L.F. Lau, and D. Nathans. 1988. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. *Proc. Natl. Acad. Sci. USA.* 85:7857-7861.
18. Lim, R.W., B.C. Barnum, and H.R. Herschman. 1987. Cloning of tetra-decanoyl phorbol ester-induced "primary response" sequences and their expression in density-arrested Swiss 3T3 cells and a TPA nonproliferative variant. *Oncogene.* 1:263-270.
19. Seyfert, V.L., S. McMahan, W. Glenn, X. Cao, V.P. Sukhatme, and J.G. Monroe. 1990. *Egr-1* expression in surface Ig-mediated B cell activation: kinetics and association with protein kinase C activation. *J. Immunol.* 145:3647-3653.
20. Carman, J.A., and J.G. Monroe. 1995. The EGR1 protein contains a discrete transcriptional regulatory domain whose deletion results in a truncated protein that blocks EGR1-induced transcription. *DNA Cell Biol.* 14:581-589.

21. Gashler, A.L., S. Swaminathan, and V.P. Sukhatme. 1993. A novel repression module, an extensive activation domain, and a bipartite nuclear localization signal defined in the immediate-early transcription factor Egr-1. *Mol. Cell. Biol.* 13:4556–4571.
22. Russo, M.W., C. Matheny, and J. Milbrandt. 1993. Transcriptional activity of the zinc finger protein NGFI-A is influenced by its interaction with a cellular factor. *Mol. Cell. Biol.* 13:6858–6865.
23. Kramer, B., A. Meichle, G. Hensel, P. Carnay, and M. Kronke. 1994. Characterization of an Krox-24/Egr-1-responsive element in the human tumor necrosis factor promoter. *Biochim. Biophys. Acta.* 1219:413–421.
24. Molnar, G., A. Crozat, and A.B. Pardee. 1994. The immediate-early gene *EGR-1* regulates the activity of the thymidine kinase promoter at the G0-to-G1 transition of the cell cycle. *Mol. Cell. Biol.* 14:5242–5248.
25. Thiel, G., S. Schoch, and D. Petersohn. 1994. Regulation of synapsin I gene expression by the zinc finger transcription factor zif268/egr-1. *J. Biol. Chem.* 269:15294–15301.
26. Ackerman, S.L., A.G. Minden, G.T. Williams, C. Bobnis, and C.-Y. Yeung. 1991. Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter. *Proc. Natl. Acad. Sci. USA.* 88:7523–7527.
27. Williams, G.T., and L.F. Lau. 1993. Activation of the inducible orphan receptor gene *nur77* by serum growth factors: dissociation of immediate-early and delayed-early responses. *Mol. Cell. Biol.* 13:6124–6136.
28. Mutero, A., S. Camp, and P. Taylor. 1995. Promoter elements of the mouse acetylcholinesterase gene: transcriptional regulation during muscle differentiation. *J. Biol. Chem.* 270:1866–1872.
29. McMahon, S.B., A. Norvell, K.J. Levine, and J.G. Monroe. 1995. Transient transfection of murine B lymphocyte blasts as a method for examining gene regulation in primary B cells. *J. Immunol. Methods.* 179:251–259.
30. Wechsler, R.J., and J.G. Monroe. 1995. *src*-family tyrosine kinase *p55^{lck}* is expressed in murine splenic B cells and is activated in response to antigen receptor cross-linking. *J. Immunol.* 154:3234–3244.
31. Ballantyne, C.M., J. Sligh, Jr., X.Y. Dai, and A.L. Beaudet. 1992. Characterization of the murine *Icam-1* gene. *Genomics.* 14:1076–1080.
32. McMahon, S.B., and J.G. Monroe. 1995. A ternary complex factor-dependent mechanism mediates induction of *egr-1* through selective serum response elements following antigen receptor cross-linking in B lymphocytes. *Mol. Cell. Biol.* 15:1086–1093.
33. Horton, R.M., Z. Cai, S.N. Ho, and L.R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques.* 8:528–535.
34. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.
35. Seyfert, V.L., V.P. Sukhatme, and J.G. Monroe. 1989. Differential expression of a zinc finger-encoding gene in response to positive versus negative signaling through receptor immunoglobulin in murine B lymphocytes. *Mol. Cell. Biol.* 9:2083–2088.
36. Xie, H., T.C. Chiles, and T.L. Rothstein. 1993. Induction of CREB activity via the surface Ig receptor of B cells. *J. Immunol.* 151:880–889.
37. Chiles, T.C., J. Liu, and T.L. Rothstein. 1991. Cross-linking of surface Ig receptors on murine B lymphocytes stimulates the expression of nuclear tetradecanoyl phorbol acetate-response element-binding proteins. *J. Immunol.* 146:1730–1735.
38. Henthorn, P., P. Zervos, M. Raducha, H. Harris, and T. Kadesch. 1988. Expression of a human placental alkaline phosphatase gene in transfected cells: use as a reporter for studies of gene expression. *Proc. Natl. Acad. Sci. USA.* 85:6342–6346.
39. Seyfert, V.L., S.B. McMahon, W.D. Glenn, A.J. Yellen, V.P. Sukhatme, X. Cao, and J.G. Monroe. 1990. Methylation of an immediate-early inducible gene as a mechanism for B cell tolerance induction. *Science (Wash. DC).* 250:797–800.
40. Sukhatme, V.P., X. Cao, L.C. Chang, C.-H. Tsai-Morris, D. Stamenkovich, P.C.P. Ferreira, D.R. Cohen, S.A. Edwards, T.B. Shows, T. Curran, et al. 1988. A zinc finger-encoding gene coregulated with *c-fos* during growth and differentiation, and after cellular depolarization. *Cell.* 53:37–43.
41. Parwardhan, S., A. Gashler, M.G. Siegel, L. C. Chang, L.J. Joseph, T.B. Shows, M.M. Le Beau, and V.P. Sukhatme. 1991. *EGR3*, a novel member of the *Egr* family of genes encoding immediate-early transcription factors. *Oncogene.* 6:917–928.
42. Joseph, L.J., M.M. Le Beau, G.A.J. Jamieson, S. Acharya, T.B. Shows, J.D. Rowley, and V.P. Sukhatme. 1988. Molecular cloning, sequencing, and mapping of *EGR2*, a human early growth response gene encoding a protein with “zinc-binding finger” structure. *Proc. Natl. Acad. Sci. USA.* 85:7164–7168.
43. Crosby, S.D., R.A. Veile, H. Donis-Keller, J.M. Baraban, R.V. Bhat, K.S. Simburger, and J. Milbrandt. 1992. Neural-specific expression, genomic structure, and chromosomal localization of the gene encoding the zinc-finger transcription factor NGFI-C. *Proc. Natl. Acad. Sci. USA.* 89:4739–4743.
44. Saffer, J.D., S.P. Jackson, and M.B. Annarella. 1991. Developmental expression of Sp1 in the mouse. *Mol. Cell. Biol.* 11:2189–2199.
45. Sligh, J., Jr., C.M. Ballantyne, S.S. Rich, H.K. Hawkins, C.W. Smith, A. Bradley, and A.L. Beaudet. 1993. Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. USA.* 90:8529–8533.
46. Xu, H., J.A. Gonzalo, Y. St Pierre, I.R. Williams, T.S. Kupper, R.S. Cotran, T.A. Springer, and J.C. Gutierrez-Ramos. 1994. Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. *J. Exp. Med.* 180:95–109.
47. McMahon, S.B., and J.G. Monroe. 1995. Activation of the *p21ras* pathway couples antigen receptor stimulation to induction of the primary response gene *egr-1* in B lymphocytes. *J. Exp. Med.* 181:417–422.
48. Cornelius, L.A., J.T. Taylor, K. Degitz, L.J. Li, T.J. Lawley, and S.W. Caughman. 1993. A 5′ portion of the *ICAM-1* gene confers tissue-specific differential expression levels and cytokine responsiveness. *J. Invest. Dermatol.* 100:753–758.
49. Ledebur, H.C., and T.P. Parks. 1995. Transcriptional regulation of the intercellular adhesion molecule-1 gene by inflammatory cytokines in human endothelial cells: essential roles of a variant NF- κ B site and p65 homodimers. *J. Biol. Chem.* 270:933–943.
- 49a. Maltzman, J., J.A. Carman, and J.G. Monroe. 1996. A role for *EGR1* in regulation of stimulus-dependent CD44 tran-

- scription in B lymphocytes. *Mol. Cell. Biol.* In press.
50. Scheinman, R.I., A. Gualberto, C.M. Jewell, J.A. Cidlowski, and A.S.J. Baldwin. 1995. Characterization of mechanisms involved in transrepression by activated glucocorticoid receptors. *Mol. Cell. Biol.* 15:943–953.
 51. Stein, B., and A.S.J. Baldwin. 1993. Distinct mechanisms for regulation of the interleukin-8 gene involve synergism and cooperativity between C/EBP and NF- κ B. *Mol. Cell. Biol.* 13:7191–7198.
 52. John, S., R.B. Reeves, J.X. Lin, R. Child, J.M. Leiden, C.B. Thompson, and W.J. Leonard. 1995. Regulation of cell-type-specific interleukin-2 receptor alpha-chain gene expression: potential role of physical interactions between Elf-1, HMG-I(Y), and NF-kappa B family proteins. *Mol. Cell. Biol.* 15:1786–1796.
 53. Gonzalez, G.A., and M.R. Montminy. 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell.* 59:675–680.
 54. Lee, C.Q., Y. Yun, J.P. Hoeffler, and J.F. Habener. 1990. Cyclic-AMP-responsive transcriptional activation of CREB-327 involves interdependent phosphorylated subdomains. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:4455–4465.
 55. Hunter, T., and M. Karin. 1992. The regulation of transcription by phosphorylation. *Cell.* 70:375–387.
 56. Lemaire, P., C. Vesque, J. Schmitt, H. Sunnenberg, R. Frank, and P. Charney. 1990. The serum-inducible mouse gene Krox-24 encodes a sequence-specific transcriptional activator. *Mol. Cell. Biol.* 10:3456–3467.
 57. Hyun, S.W., K. Park, Y.S. Lee, Y.I. Lee, and S.-J. Kim. 1994. Inhibition of protein phosphatases activates P4 promoter of the human insulin-like growth factor II gene through the specific promoter element. *J. Biol. Chem.* 269: 364–368.
 58. Cao, X.M., R.A. Koski, A. Gashler, M. McKiernan, C.F. Morris, R. Gaffney, R.V. Hay, and V.P. Sukhatme. 1990. Identification and characterization of the Egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals. *Mol. Cell. Biol.* 10:1931–1939.