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Role of EGR1 in Regulation of Stimulus-Dependent CD44 Transcription in B Lymphocytes

JONATHAN S. MALTZMAN, JULIE A. CARMAN, AND JOHN G. MONROE*

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

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The immediate-early gene *egr*-1 encodes a transcription factor (EGR1) that links B-cell antigen receptor (BCR) signals to downstream activation events through the regulation of previously unidentified target genes. Here we identify the gene encoding the lymphocyte homing and migration protein CD44 as a target of EGR1 regulation in B cells. BCR-induced increases in CD44 mRNA expression and transcription levels are shown to occur in EGR1-expressing but not in nonexpressing subclones of the B-cell line WEHI-231. Kinetics of *egr*-1 transcription and the appearance of nuclear EGR1 protein precede CD44 induction and occur within 30 min after stimulation in the EGR1-expressing subclone. A single EGR1 binding motif is demonstrated at bp −301 of the human CD44 promoter. Cotransfection of a CD44 promoter-chloramphenicol acetyltransferase reporter construct with an *egr*-1 expression vector resulted in a 6.5- to 8.5-fold induction of transcriptional activity relative to an empty expression vector. The EGR1 binding motif was shown to be necessary for stimulus-induced expression of a CD44 promoter-chloramphenicol acetyltransferase reporter construct in nontransformed B lymphocytes and was required for transactivation by an EGR1 expression vector in a B-cell line. These studies identify EGR1 as an intermediary linking BCR-derived signals to the induction of CD44. The relevance of these molecular events to BCR signal transduction and antigen-stimulated B-cell-mediated immune responses is discussed.

In vivo, antigenic stimulation of mature B lymphocytes initiates processes leading to alterations in cell surface phenotype, migration and adhesion, clonal proliferation and, ultimately, differentiation into memory and antibody-secreting plasma cells. An approach to understanding the underlying molecular basis for these changes has been to study signal transduction events associated with cross-linking of the B-cell antigen receptor (BCR) of mature B lymphocytes. It is well established that BCR cross-linking leads to alterations in biochemical second messengers (4) followed by the induction of immediate-early-gene-encoded transcription factors (28, 39, 42); the latter are thought to function by translating second-messenger pathways into relevant B-cell responses through the coordinate regulation of secondary (delayed) response genes (35).

CD44 (Pgp-1) is a cell surface glycoprotein expressed on multiple cell types, including both B and T lymphocytes (2). Cell surface levels are upregulated on murine B lymphocytes following stimulation through the BCR (5). CD44 has recently been shown to be the cellular receptor for hyaluronic acid (HA) (2, 40, 63). Binding to HA has been implicated in the migration of lymphocytes to and their retention in peripheral lymphoid tissues (45). BCR-induced changes in CD44 expression (44) and posttranslational modification (22, 27, 32) are proposed to facilitate B-cell migration to and retention in secondary lymphoid organs where they then encounter and receive secondary activation signals from T helper cells.

The immediate-early gene *egr*-1 (60, 61) (also known as NGFI-A [38], Krox-24 [30], and *Zif*268 [13]) encodes a nuclear protein (EGR1) that is the prototypic member of a family of transcription factors sharing a conserved zinc finger DNA binding motif (6, 41). Although these proteins bind with high affinity to the consensus 9-bp site 5′-GCG (T/G)GG GCG-3′ (12, 31), binding to motifs differing in 1 or 2 bp has also been reported (41, 49). In addition, flanking sequences can have an effect on binding affinity (62). We have previously shown that *egr*-1 expression is induced within 30 min following BCR cross-linking of splenic B lymphocytes (55). Studies using antisense oligonucleotides have suggested that *egr*-1 expression is necessary for the in vitro activation of murine splenic B lymphocytes in response to BCR cross-linking (43). The EGR1 protein has been shown to be a transcriptional activator in B cells (8) as well as other cells (17, 51), and its role in BCR-initiated responses is thought to be due to its ability to regulate the expression of specific activation-associated genes in B cells.

Sequence analysis of the CD44 5′ flanking region identified the presence of two potential EGR1 binding sites upstream of the proposed transcription start site of this gene. Our unpublished data (now shown herein) revealed an increase in CD44 message levels between 2 and 4 h after BCR stimulation. Thus, the kinetics of induction of the CD44 gene are consistent with its being among the class of delayed response genes in BCR-initiated B-cell responses. The kinetics of induction of CD44 by BCR cross-linking and the presence of potential EGR1 binding sites suggested the possibility that CD44 transcription is regulated by EGR1 during BCR signaling. In this study, we have investigated the role of EGR1 in regulating CD44 induction in response to BCR signaling and a pharmacologic agent (the phorbol ester phorbol myristate acetate [PMA]) which mimics specific aspects of BCR signal transduction. Our studies indicate that CD44 is a target gene of EGR1 and, therefore, suggest that EGR1 acts to link BCR-mediated signals to the transcriptional regulation of a mediator of B-cell migration and homing.

* Corresponding author. Mailing address: Department of Pathology and Laboratory Medicine, Room 538A, Clinical Research Building, 415 Curie Blvd., Philadelphia, PA 19104. Phone: (215) 898-2873. Fax: (215) 573-2014. Electronic mail address: MonroeJ@mail.med.upenn.edu.

MATERIALS AND METHODS

Cell culture and isolation of murine B lymphocytes. The WEHI-231 B-cell lymphoma (American Type Culture Collection) and the limiting dilution subclones WEHI-231.7 and WEHI-231.1F1 (hereafter referred to as 231.7 and 1F1, respectively) were maintained in Dulbecco's modified Eagle's medium, high glucose, supplemented with 10% fetal calf serum (defined; Hyclone), SerExtend (Hana Biologicals, Berkeley, Calif.), 3 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol. Cells were maintained at a minimum and maximum density of 1×10^4 and 5×10^5 per ml, respectively.

B lymphocytes were isolated from spleens of BALB/c mice as previously described (37), Briefly, spleens were ground between the frosted ends of two glass slides to produce single-cell suspensions. Following the depletion of T cells by treatment with anti-Thy1.2 antibody and complement and erythrocytes by lysis with Grey'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% immunoglobulin M-positive (IgM^+) $B220^+$ cells. Cells were then incubated at 37°C, 5% CO_2, for 72 h in B-cell assay medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, OPI supplement [Gibco BRL, Gaithersburg, Md.], nonessential amino acids [Gibco BRL], 100 μg of penicillin per ml, 100 μg of streptomycin per ml, and 5×10^{-5} 2-mercaptoethanol) and 50 μg of lipopolysaccharide (LPS) (Sigma) per ml.

Plasmids, constructs, and probes. pAC-mEGR1, containing the murine *egr*-1 cDNA under the transcriptional control of the β-actin promoter, was the generous gift of D. Liebermann (Temple University) (46). pAC was constructed by excision of *egr*-1 from pAC-mEGR1 with *Sal*I and *Bam*HI; this was followed by blunt-end generation with Klenow polymerase and religation. pAC-hEGR1 was constructed by subcloning a 1.7-kb fragment of the human *egr*-1 cDNA from H-364 (59) into the *Sal*I-*Bam*HI site of pHβ-Actin-polyAneo (19).

rlcp1, containing the full-length CD44 cDNA, was the generous gift of E. Puré (5). pBlueCD44 was constructed by subcloning a 1.3-kb *Hin*dIII-*Not*I fragment containing the entire CD44 cDNA into pBluescript SK+ (Stratagene). pGAPDH, containing a 1.2-kb glyceraldehyde 3-phosphate dehydrogenase (GAPDH) insert in pBluescript SK+, was the generous gift of M. Prystowski (Albert Einstein College of Medicine, Bronx, N.Y.). pBlueEGRΔ3′, containing the *egr*-1 region between bp 1 and 1246, was constructed by subcloning of the 3.1-kb murine *egr*-1 cDNA into the *Eco*RI site of pBluescript SK+ followed by digestion with *Bam*HI and *Nde*I, blunt-end generation, and religation.

pSacBCAT, containing bp −701 to −42 of the human CD44 promoter fused upstream of the chloramphenicol acetyltransferase (CAT) reporter gene was provided by E. Shtivelman (57). The region encompassing −701 to −151 was excised with *Hin*dIII and *Pst*I and subcloned into pBluescript SK+ (Strategene). A *Hin*dIII-*Bam*HI fragment was then subcloned into *Hin*dIII-*Bam*HI-digested pBLCAT2 (18) to generate the reporter construct pBLCD44. For construction of pBLmCD44, splicing overlap PCR (25) was used to mutate the EGR1 binding motif at bp −301. The oligonucleotides used were 5′-CACGGGGCGGGTCTA GAGGG-3′ and its reverse complement. Presence of the mutation was confirmed by sequencing with a Sequenase 2.0 kit (U.S. Biochemicals, Cleveland, Ohio) according to the manufacturer's instructions. pSV_2APAP (a generous gift of T. Kadesch, University of Pennsylvania) contains the gene for the placental alkaline phosphatase (PAP) under the transcriptional control of a constitutive simian virus 40 promoter (23). pBLET-ERE contains a single EBS cloned into the *Sal*I site of pBLCAT2 as previously described (8).

Antibodies for fluorescence-activated cell sorter analysis. Analysis for relative IgM, IgD, CD45, and CD5 expression was accomplished by using the following reagent combinations. $F(ab')_2$ fragments of fluorescein isothiocyanate-conjugated goat anti-mouse μ antibody (Jackson Immunoresearch Labs, Inc., West Grove, Pa.) were used to stain for relative IgM expression. Phycoerythrin-conjugated rat anti-δ antibody (Southern Biotechnology Associates, Inc., Birmingham, Ala.) was used for IgD expression. Biotin-conjugated antibodies from the hybridoma M1/9.3.4 (American Type Culture Collection) was used in combination with phycoerythrin-conjugated streptavidin to determine CD45 levels. Finally, phycoerythrin-conjugated anti-mouse CD5 monoclonal antibody was obtained from Pharmingen. Isotype negative controls were used in all analyses.

RNA isolation and Northern RNA analysis. Cells (2.5×10^7) were placed in 50 ml of medium and equilibrated for 2 h at 37°C, 5% CO_2, for 1 to 2 h prior to stimulation. Following preincubation, 10 ng of PMA (Sigma, St. Louis, Mo.) per ml or 10 μg of goat anti-mouse μ antibody (Sigma) per ml was added for the times indicated in the text, and total RNA was isolated by using the acid guanidinium thiocyanate-phenol-chloroform method (11). Equal amounts of RNA were electrophoresed through a 1.2% formaldehyde-agarose gel and blotted to GeneScreen Plus nylon membranes (NEN/Dupont Co., Boston, Mass.), fixed by UV irradiation, and baked for 2 h at 80°C in a vacuum oven. The 3.1-kb *egr*-1 cDNA, 1.2-kb GAPDH cDNA, and rlcp1 (CD44) were labeled by incorporation of [^{32}P]dCTP by nick translation according to the instructions of the manufacturer (Gibco BRL). Following a 2- to 6-h prehybridization in 50 mM PIPES [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid); pH 6.8]–50 mM $NaPO_4$–100 mM NaCl–1 mM EDTA–5% sodium dodecyl sulfate (SDS) at 65°C, denatured probe was added and hybridization was carried out for 12 to 16 h at 65°C. The blots were washed three times at 65°C for 20 min each time with 1.0× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 5% SDS; three times, for 20 min each time, with 0.5× SSC and 0.1% SDS; and three times, for 20 min each time, with 0.2× SSC and 0.1% SDS. Quantitative analysis was carried out by using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

Electrophoretic mobility shift assays (EMSA). Exponentially growing cells were stimulated with 10 ng of PMA per ml for 2 h and washed twice in phosphate-buffered saline (PBS) at 4°C, and nuclear extracts were prepared as described previously (10, 64). Probes were labeled with T4 polynucleotide kinase according to the instructions of the manufacturer (New England Biolabs, Beverly, Mass.) and purified on a 12% native polyacrylamide gel. Binding reactions were carried out in a 20-μl volume containing 10,000 dpm of labeled probe, nuclear extract containing 10 μg of protein, 1 μg of poly(dI-dC) · poly(dI-dC) (Pharmacia, Piscataway, N.J.), 10 mM Tris (pH 7.5), 40 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 4% glycerol with or without competing oligonucleotides as indicated in the text. For antibody blocking experiments, antisera specific for EGR1 or CDK4 (Santa Cruz Biotechnology, Santa Cruz, Calif.) or protein A-adsorbed rabbit anti-EGR1 antibody (amino acids 519 to 533) were preincubated with nuclear extract and poly(dI-dC) · poly(dI-dC) for 15 min at room temperature. Following the addition of reaction buffer and labeled probe, the reaction was allowed to proceed for an additional 15 min at room temperature. Reaction mixtures 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 150 V at room temperature.

The EBS-containing probe used for Fig. 1 was excised from pBLET-ERE by digestion with *Pst*I and *Bam*HI and contains the sequence 5′-gaGGCGGGGC Gct-3′ flanked by a sequence from pBLET-ERE. Complementary oligonucleotides containing the serum response element (SRE) (5′-CACAGGATGTCCAT ATTAGGACATCTGCGT-3′), bp −301 EGR1 (5′-CACGGGGCGGGGGCA GAGGG-3′), and mutant bp −301 EGR1 (5′-CACGGGGCGGGTCTAGAG GG-3′) were synthesized (Chemistry Department, University of Pennsylvania) and annealed by heating to 100°C for 5 min in 100 mM NaCl and slowly cooling to room temperature. AP1 (5′-CGCTTGATGAGTCAGCCGGA-3′) and Sp1 (5′-ATTCGATCGGGGCGGGGCGAGC-3′) (Promega, Madison, Wis.) as well as EGR1 (5′-GGATCCAGCGGGGGCGAGCGGGGGCGA-3′) (Santa Cruz Biotechnology) were purchased as double-stranded oligonucleotides.

Nuclear run-on transcription assays. Exponentially growing cells were aliquoted into 50-ml conical tubes; equilibrated at 37°C, 5% CO_2, for 2 h; and stimulated with 10 ng of PMA per ml. At the indicated times, the cells were pelleted, washed twice with PBS at 4°C, and lysed in 1 ml of lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 8.5], 1.5 mM $MgCl_2$, 1.0 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride [PMSF], 1.5 μg each of pepstatin A, chymotrypsin, leupeptin, and antipain per ml) on ice for 15 min. Following one wash in lysis buffer, nuclei were resuspended in 1 ml of lysis buffer with 10 μg of RNase A per ml for 30 min at 4°C to remove cytoplasmic RNA. The nuclei were then washed twice in lysis buffer at 4°C and resuspended at 10^7 nuclei per 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 DTT, 0.125 mM PMSF), placed into a dry-ice–ethanol 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 $MnCl_2$, 1.2 mM DTT, 0.1 mM PMSF, 0.4 mM EDTA, 1.0 mM [each] nucleoside triphosphates, 10 mM phosphocreatine, 40 U of RNasin (Promega), 2.5 μM UTP, 200 μCi of [^{32}P]UTP); this was followed by the addition of 1.5 U of RNase-free DNase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and incubation at 26°C for an additional 5 min. Reactions were terminated by the addition of 1 ml of guanidinium thiocyanate-phenol-sodium acetate, pH 4.0, and RNA was extracted as described previously (9). Unincorporated nucleotides were removed by two successive ammonium acetate (2.5 M)–ethanol precipitations. Prehybridized GeneScreen Plus membranes containing 5 μg of denatured pBluescript SK+ with or without inserts containing murine CD44 (pBlueCD44), murine *egr*-1 (pBlueEGRΔ3′), or murine GAPDH (pGAPDH) were hybridized for 65 h at 42°C with 1.2×10^7 cpm of nuclear run-on products in 1 ml of 50% formamide–5× SSC–50 mM $NaPO_4$ (pH 7.0)–0.1% $NaPP_i$–1% SDS–1 mg of heparin per ml–100 μg of sheared herring sperm DNA per ml. The membranes were washed for 15 min at 22°C (2× SSC–0.1% SDS) and for 15 minutes at 68°C (0.1× SSC–1% SDS). Quantitative analysis was done as described above.

Transient transfection and reporter assays. 231.7 cells were transfected by electroporation. Exponentially growing 231.7 cells were washed twice in PBS (Ca^{2+} and Mg^{2+} free) at 4°C and resuspended at 10^7 cells per ml in PBS. For electroporation, 0.6 ml of the cell suspension was transferred into precooled 0.4-cm-wide cuvettes (Bio-Rad Laboratories, Richmond, Calif.). Following the addition of plasmid DNA, the cells were incubated on ice for 10 min, pulsed with the GenePulser apparatus with capacitance extender (Bio-Rad Laboratories) at 270 V and 960 μF of capacitance, incubated on ice for a further 10 min, and transferred into 30 ml of fresh culture medium. pSV_2APAP was cotransfected to control for transfection efficiency. Forty-eight hours following transfection, the cells were harvested and CAT and placental alkaline phosphatase assays were performed as described previously (23, 55). CAT activity (percent conversion) was quantitated by PhosphorImager analysis as described above and calculated as a ratio of acetylated chloramphenicol to total chloramphenicol (acetylated plus nonacetylated).

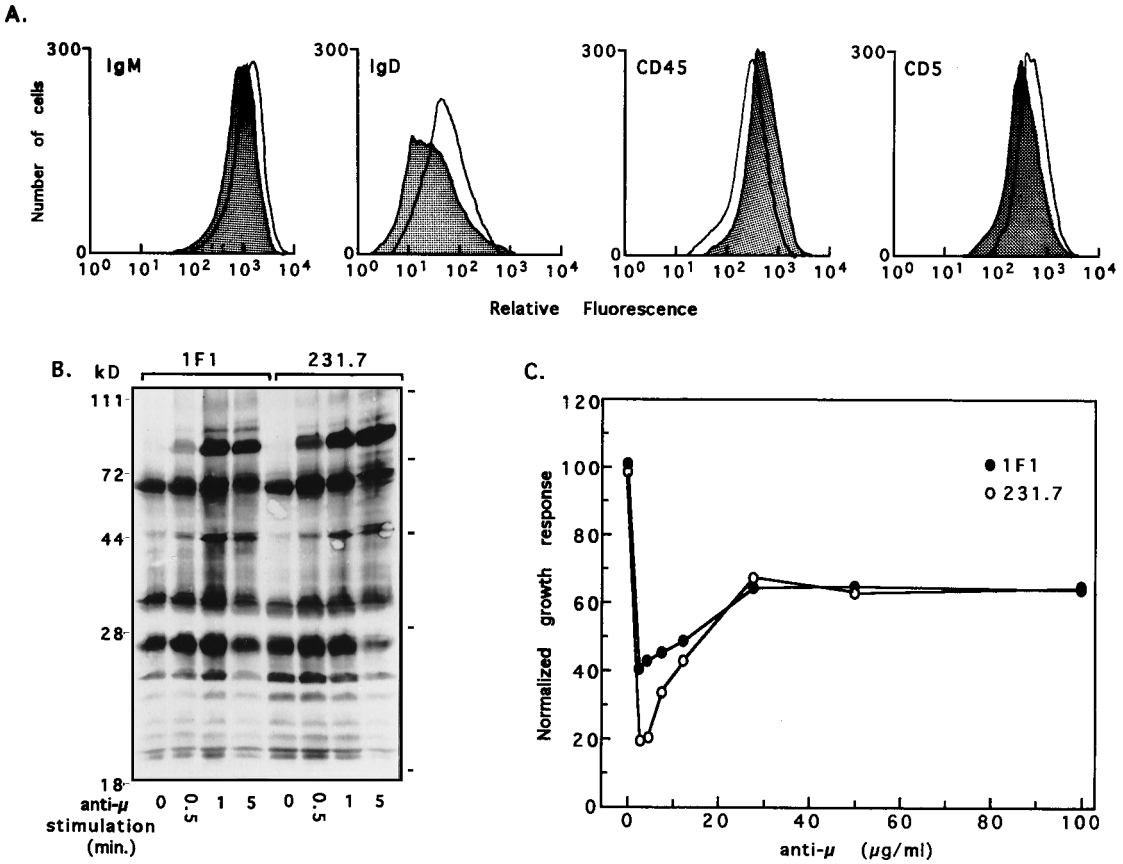

FIG. 1. Functional characterization of the 1F1 and 231.7 subclones. (A) FACS analysis for relative IgM, IgD, CD45, and CD5 surface expression. IgM levels were measured as described in Materials and Methods. Isotype-matched negative controls were performed for each analysis (data not shown) and used to normalize the relative level of each marker. Shaded curve, 231.7 cells; unshaded curve, 1F1 cells. (B) Phosphotyrosine immunoblotting of 1F1 and 231.7 subclones following stimulation with 50 μg of anti-μ antibody per ml. Whole-cell lysates after the indicated period of stimulation were prepared by detergent lysis with 0.5% Triton X-100 with proteases and tyrosine phosphatase inhibitors. Cellular proteins were separated on SDS–10% polyacrylamide gels, and tyrosine phosphoproteins were detected by 4G10 immunoblotting and enhanced chemiluminescence by using a horseradish peroxidase-coupled goat anti-mouse secondary antibody (Amersham). (C) Growth response of 1F1 and 231.7 cells after anti-μ antibody stimulation. Cells were stimulated with the indicated dose of anti-μ antibody for 24 h, at which time the cultures were pulsed with 1 μCi of [^{3}H]thymidine per well and harvested 16 h later. Results depict levels of incorporation of triplicate cultures normalized to levels of incorporation by wells containing no antibody (100).

Primary lymphocytes were transfected as described previously (37). Briefly, 5×10^7 LPS blasts were washed once in STBS and resuspended at 1×10^7 cells per ml in STBS containing 500 μg of DEAE-dextran (Pharmacia) per ml and the indicated plasmid DNA for 30 min at 37°C. The cells were then washed once in STBS, resuspended at 5×10^6 cells per ml in fresh B-cell assay medium, and incubated at 37°C, 5% CO_2. After 24 h, the cells were split into two equal groups and either were left unstimulated or were stimulated with 10 ng of PMA per ml. Following an additional 24 h of incubation, the cells were harvested and assayed for CAT activity.

RESULTS

Subclones of the WEHI-231 B-cell lymphoma line differ in their expression of EGR1. We have developed a system which can be used to examine the involvement of EGR1 in the transcriptional regulation of other genes. The system relies on two subclones of the WEHI-231 B-cell lymphoma which differ in expression of EGR1. Previous studies in our laboratory identified a subclone of WEHI-231 that was unable to express the *egr*-1 gene in response to BCR cross-linking or exposure to the phorbol ester PMA (56). Silencing of *egr*-1 gene transcription in this line, 231.7, is due to methylation of the *egr*-1 locus (56). More recently, we have derived another subclone, 1F1, in which induction of *egr*-1 is observed following BCR cross-linking with anti-μ antibodies.

The usefulness of this system for analysis of EGR1 target genes depends upon how similar these subclones are with respect to parameters other than *egr*-1 inducibility. In this regard, 231.7 and 1F1 are very similar with respect to their expression of surface IgM, IgD, CD45, and CD5 (Fig. 1). Although there are slight variations in the expression of these surface proteins, it is unlikely that they are sufficient to affect differences in the response of these cells to antigen receptor (IgM in these studies) stimulation. In fact, anti-μ antibody-induced tyrosine kinase activities and the growth responses of these two subclones of WEHI-231 to BCR cross-linking are nearly identical (Fig. 1B and C, respectively). WEHI-231 undergoes cell cycle arrest (53) and apoptosis (3, 21) in response to anti-μ antibody stimulation. Both the 1F1 and 231.7 subclones showed equivalent inhibition of proliferation by anti-μ antibody stimulation.

While 1F1 and 231.7 cells differ in their inducible expression of *egr*-1 in response to anti-μ antibody stimulation (Fig. 2A), they do not differ in their inducible expression of other immediate-early genes. As shown in Fig. 2B and C, both 1F1 and 231.7 cells show induction of *egr*-2, *junB*, and c-*myc* in response to anti-μ antibody stimulation. In addition, c-*fos* induction was observed in both cells following anti-μ antibody stimulation (data not shown). Of particular interest is the *egr*-2 response,

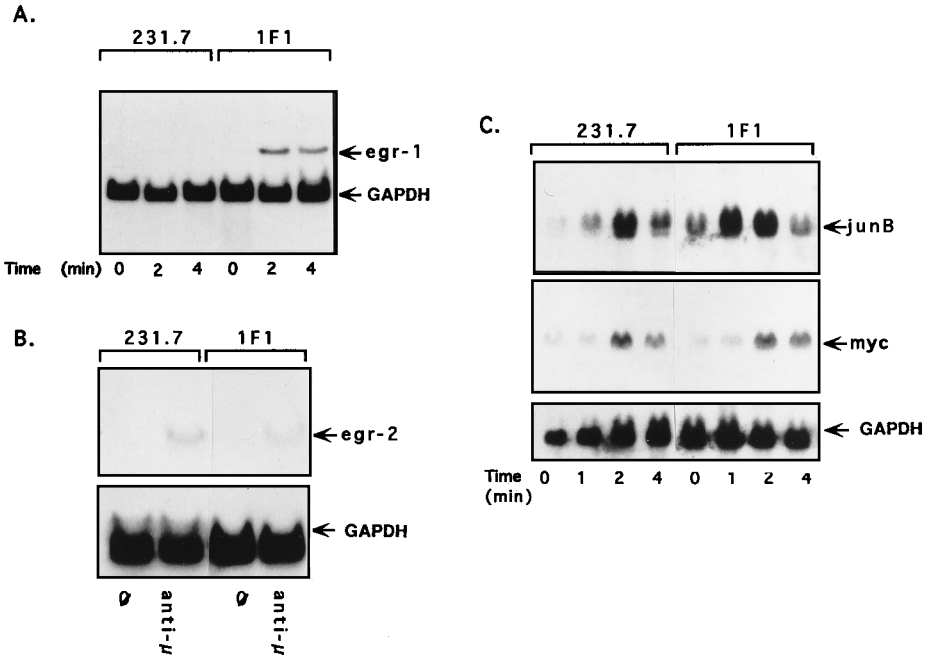

FIG. 2. mRNA levels of *egr*-1, *egr*-2, *junB*, and c-*myc* in 1F1 and 231.7 cells after anti-μ antibody stimulation. Cells were cultured for the indicated period of time with 50 μg of anti-μ antibodies per ml, and total RNA was isolated. Northern blots were hybridized with labeled probes from murine cDNAs of *egr*-1 (A), *egr*-2 (1 h) (B), *junB* (C), c-*myc* (C) or GAPDH as a control for RNA loading. Ø, unstimulated controls.

which indicates that the differential inducibility of *egr*-1 in 1F1 and 231.7 cells does not extend universally to other members of this family. Thus, while we cannot formally rule out that the 1F1 and 231.7 subclones of WEHI-231 differ in aspects other than *egr*-1 inducibility, they nonetheless appear to be very similar. For this reason, we considered that this would be a useful system to identify and evaluate EGR1 target genes in stimulated B lymphocytes.

Differential inducibility of *egr*-1 in 1F1 and 231.7 B cells was extended to include stimulation with the phorbol ester analog PMA. As with anti-μ antibody stimulation, Northern analysis of *egr*-1 revealed a lack of inducible message in the 231.7 subclone, whereas PMA stimulation (Fig. 3A) of 1F1 cells led to a rapid (within 30 min) and transient induction of *egr*-1 mRNA. By bypassing the antigen receptor and directly initiating BCR-associated signaling pathways leading to *egr*-1 gene induction (36), PMA is believed to model IgM-induced *egr*-1. In so doing, we have observed PMA to be a more efficient inducer of *egr*-1 expression than anti-μ antibody. Finally, the kinetics of PMA-induced *egr*-1 expression in these cells closely parallel the induction of *egr*-1 in primary B lymphocytes by PMA or anti-μ antibody (55).

As would be expected from Northern analysis (Fig. 3A), stimulated 1F1 cells expressed the EGR1 protein, whereas 231.7 cells do not. Nuclear extracts of PMA-stimulated 1F1 cells but not 231.7 cells exhibited EGR1 binding to a probe containing a consensus EGR1 binding site that was specifically inhibited by an oligonucleotide containing two consensus binding sites (EGR1 in Fig. 3B). An oligonucleotide containing an irrelevant binding site, the c-*fos* SRE, had no effect on this protein-DNA complex.

Only a single complex was inhibited by the unlabeled EGR1 site-containing oligonucleotide, suggesting either that only one EGR family member was present in these extracts or that if multiple proteins bind specifically, they exhibit identical mobilities under the gel conditions used in this experiment. To determine if this complex was due solely to EGR1 binding, we performed binding reactions in the presence of an antiserum raised against a C-terminal peptide of EGR1 that is unique to EGR1 among members of this family (15, 26, 47, 60). Preincubation with anti-EGR1 antiserum inhibited EGR1 site-specific binding in a dose-dependent manner. This result, taken together with the oligonucleotide competition data, indicated that extracts prepared from PMA-stimulated 1F1 cells do not contain detectable EGR2, EGR3, EGR4, or WT1 binding activities. Thus, although they express *egr*-2 mRNA at detectable, albeit low, levels (Fig. 2B), protein levels are insufficient to form detectable complexes, indicating that EGR1 is the predominant DNA-binding protein from this family that is expressed in this system. In summary, while 1F1 cells expressed both *egr*-1 message and protein following PMA and anti-μ antibody stimulation, 231.7 cells do not manifest detectable expression following stimulation by these agents.

Increased CD44 mRNA expression by PMA or BCR stimulation correlates with EGR1 inducibility and is regulated at the level of transcription. To evaluate the possibility that EGR1 is involved in the inducible transcription of CD44, we compared CD44 mRNA levels in 231.7 and 1F1 cells in response to BCR signaling. Three distinct CD44 transcripts (corresponding to published lengths of 1.3, 2.7, and 3.9 kb [5]) were constitutively expressed at low levels in both cell lines (Fig. 4A). The presence of multiple transcripts is thought to be the result of alternative splicing (54) and differential polyadenylation (57). Stimulation through the BCR with anti-μ antibodies stimulated a marked increase in the levels of all three CD44 mRNAs in 1F1 cells. An increase was also detectable in the 231.7 line, but the magnitude of this increase was much less than that observed with 1F1 cells. In this experiment, inducible expression peaked at 4 h. To control for general increases in transcription and RNA loading, message levels of the housekeeping gene GAPDH were measured. GAPDH mRNA levels did not vary significantly between 1F1 and 231.7 cells nor

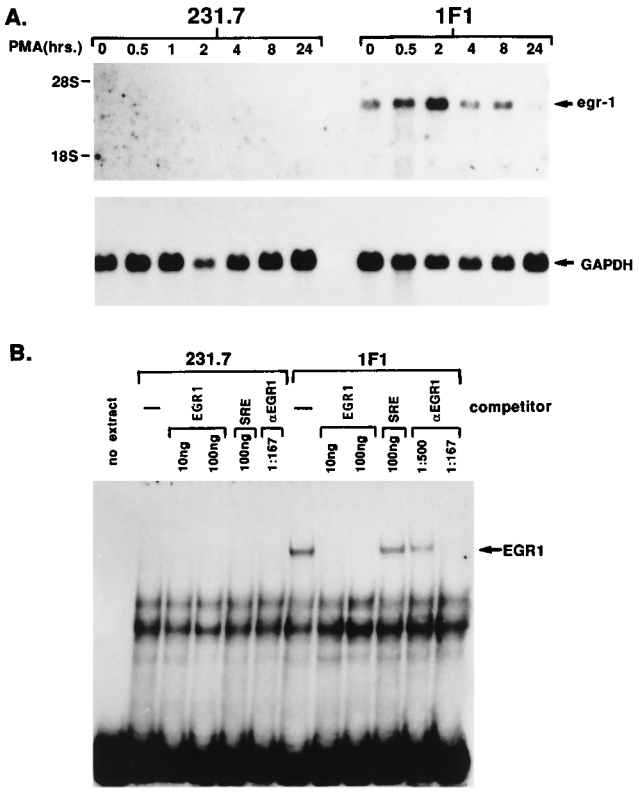

FIG. 3. *egr*-1 message and protein expression are induced by PMA in 1F1 but not in 231.7 cells. (A) Time course of *egr*-1 RNA induction. Ten micrograms of total cellular RNA isolated from 231.7 and 1F1 cells at the indicated times following exposure to PMA (10 ng/ml) was subjected to Northern blot analysis for *egr*-1 and GAPDH. The locations of 28S and 18S rRNA bands are indicated. (B) Gel mobility shift assays were performed with 8 μg of nuclear extracts prepared from 231.7 and 1F1 cells stimulated for 2 h with 10 ng of PMA per ml. The 65-bp ^{32}P-labeled probe used contains a single EGR1 consensus binding site and flanking plasmid DNA. Unlabeled oligonucleotide or anti-EGR1 antiserum was preincubated with nuclear extracts at 4°C for 15 min, the probe was added, binding was allowed to proceed for an additional 15 min at room temperature, and the reactions were separated through a 4% polyacrylamide–0.5× TBE gel.

following stimulation of these cells. Similar differential induction of CD44 was also seen when PMA was used as a stimulus (data not shown). Therefore, induction of CD44 message following either PMA or anti-μ antibody stimulation correlates with the capacity of these cells to express EGR1.

The differential levels of CD44 RNA detected by Northern analysis could be due either to increased rates of transcription or to differences in CD44 RNA stability or to both. A direct role for the EGR1 transcription factor implies effects at the level of CD44 transcription. To evaluate the importance of induced transcription of the CD44 gene to the increase in mRNA levels, measurements of de novo transcription were performed by using nuclear run-on assays.

Transcription of CD44 in 1F1 cells was induced within 1 h of PMA stimulation (Fig. 5A). Consistent with the kinetics of steady-state RNA induction (Fig. 4 and data not shown), the rate of CD44 transcription peaked between 1 and 3 h following stimulation. To compare levels of transcription at different times between the two cell lines, we quantitated the band intensities and normalized these values to the transcription of GAPDH (Fig. 5B). Stimulation indices indicated that CD44 transcription was induced approximately 5-fold within 2 h in 1F1 cells, while less than 1.3-fold induction was seen at any time with 231.7 cells (Fig. 5B). While not ruling out the involvement of increased message stability as a component of the increase in *egr*-1 mRNA levels after PMA stimulation of 1F1 B cells, these results clearly establish a stimulus-induced increase in the rate of *egr*-1 gene transcription.

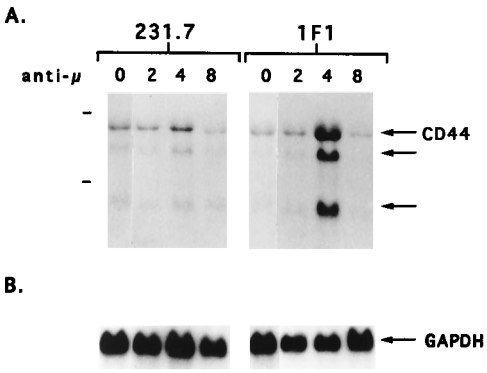

FIG. 4. Comparison of anti-μ antibody-induced CD44 mRNA levels in 231.7 and 1F1 cells. Total RNA isolated from 231.7 and 1F1 cells following stimulation with 10 μg of goat anti-μ antibody per ml for the times indicated (in hours) was subjected to Northern analysis for CD44 (A) and GAPDH (B). CD44-specific transcripts corresponding to 3.9-, 2.7-, and 1.3-kb species are indicated by arrows. The migration of 28S and 18S rRNA bands is indicated by the dashes on the left.

If EGR1 protein regulates the transcription of CD44, we

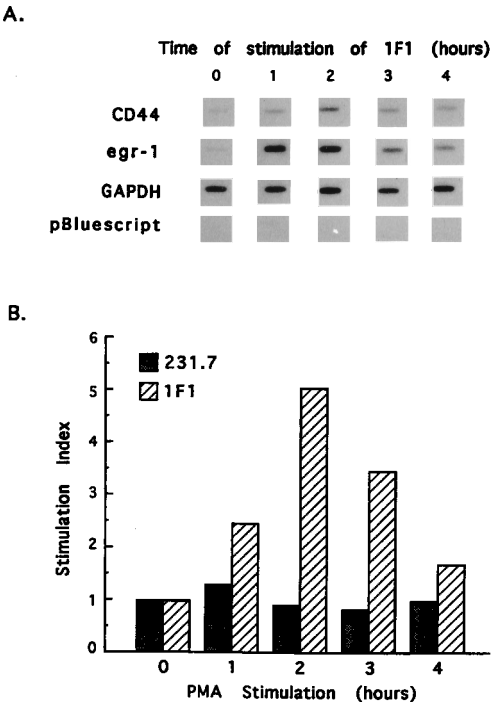

FIG. 5. Differential induction of CD44 transcriptional initiation in 1F1 and 231.7 cells. (A) Nuclei were isolated from 1F1 cells that had been stimulated with 10 ng of PMA per ml for the times indicated and were used as templates to produce ^{32}P-labeled nuclear run-on transcription probes as described in Materials and Methods. Five micrograms each of pBluescript SK+ vectors containing either CD44, *egr*-1, or GAPDH or containing no insert were immobilized onto GeneScreen Plus membranes. The run-on transcription products were hybridized to the membranes. (B) Quantitative analysis of PMA-stimulated CD44 transcription in 231.7 and 1F1 cells was performed by using a PhosphorImager (Molecular Dynamics). CD44 band intensities from the hybridized filters in panel 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.

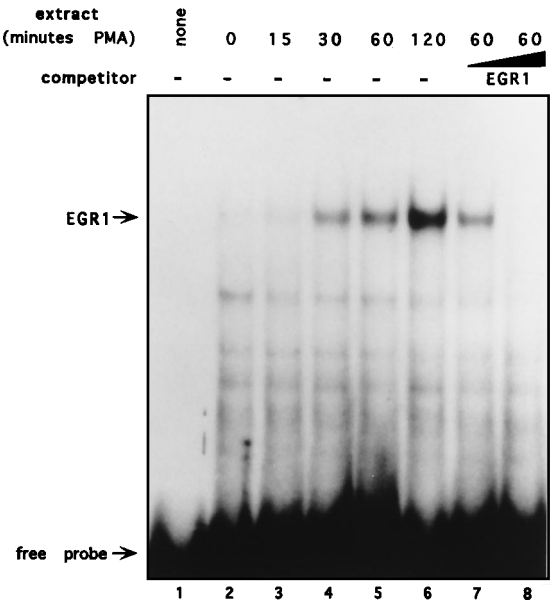

FIG. 6. PMA rapidly induces nuclear EGR1 protein. EMSA were carried out with 5 μg of nuclear extracts from 1F1 cells which had been stimulated with PMA for the times indicated above each lane. The extracts were incubated with a ^{32}P-labeled oligonucleotide probe containing two consensus EGR1 binding sites for 15 min; this was followed by separation on a 4% polyacrylamide–0.5× TBE gel and autoradiography. In lanes 7 and 8, a 20- or 100-fold molar excess of unlabeled competing oligonucleotides was preincubated with nuclear extracts from 60-min-stimulated 1F1 cells prior to the addition of labeled probe.

would expect that *egr*-1-specific transcription would precede that of CD44. The rate of *egr*-1 transcription was induced by greater than 18-fold within 1 h following PMA treatment of 1F1 cells (Fig. 5A). Among the times examined, maximal levels of *egr*-1 transcription were attained at 1 h (versus 2 h for CD44), decreasing over the next 3 h. As expected, *egr*-1 transcription in 231.7 cells was not detectable (data not shown).

The kinetics of nuclear EGR1 protein correlate with CD44 transcription. Although the kinetics of *egr*-1 transcription correlate with the transcription of CD44, the relevance of EGR1 as a transcriptional activator of the CD44 gene depends on the kinetics of EGR1 protein available in the nucleus. To address whether EGR1 protein is induced with appropriate kinetics to regulate CD44 transcription (i.e., within 1 to 2 h), 1F1 cells were stimulated with PMA for varying periods of time and nuclear extracts were used for EMSA with an oligonucleotide probe containing two tandem EGR1 consensus binding motifs (Fig. 6). No detectable EGR1 binding was seen with unstimulated 1F1 cells or with cells stimulated for 15 min. However, nuclear EGR1 protein could be detected beginning within 30 min following PMA stimulation, with binding activity continuing to increase until 2 h following stimulation. The kinetics of EGR1 binding activity, therefore, closely parallel the induction of CD44 specific transcription (Fig. 5).

EGR-1 binds with low affinity to a site at bp −301 of the CD44 promoter region. While EGR1 is present in the nucleus with appropriate kinetics to regulate CD44 transcription, this regulation presumes the ability of the EGR1 protein to specifically interact with the CD44 promoter. Sequence analysis of the human CD44 promoter indicated two potential EGR1 binding motifs centered at bp −600 and −301, relative to the translation start site. Since each of these elements differs from the consensus binding site by at least 1 bp, we carried out in vitro binding studies to determine binding of EGR1 to DNA fragments containing each of these elements. Preliminary EMSA experiments using large (approximately 100-bp) fragments which included the potential EGR1 motifs showed an EGR1-specific band shift of a fragment containing the bp −301 motif but not the bp −600 motif (34a), thereby ruling out the site at bp −600 as a potential mediator of EGR1-regulated CD44 expression.

Because the motif located at bp −301 is a nonconsensus binding motif, further studies using a synthetic oligonucleotide were carried out to confirm EGR1 binding and to compare the affinity of EGR1 for this sequence to that of the consensus. Our system utilizing the 231.7 and 1F1 cell lines offers the advantage of determining EGR1 binding by using murine nuclear extracts rather than in vitro-synthesized or bacterially expressed protein. If EGR1 binds to the DNA probe, a shifted band unique to nuclear extracts of stimulated 1F1 cells should be present.

Nuclear extracts from PMA-stimulated 231.7 and 1F1 cells were used in gel mobility shift assays with a labeled oligonucleotide probe spanning bp −288 to −307 of the CD44 promoter. Incubation of the probe with stimulated extracts from 231.7 cells resulted in two shifted complexes (Fig. 7, lane 2). The high-mobility complex was not inhibited by excess unlabeled probe and was attributed to nonspecific binding (data not shown). Competition analysis with unlabeled oligonucleotides for Sp1 and EGR1 indicated that the low-mobility complex was due to Sp1 binding (Fig. 7, lanes 2 to 4). Use of stimulated extracts from 1F1 cells resulted in the appearance of an additional low-mobility complex (Fig. 7, lane 5). This complex was specifically inhibited by EGR1 consensus oligonucleotides but not Sp1 or AP1 oligonucleotides (Fig. 7, compare lane 7 and lanes 6 and 8) in other experiments and comigrated with a band specifically inhibited with an EGR1-specific antibody (data not shown). Because the only detectable EGR family protein present in 1F1 extracts that binds to

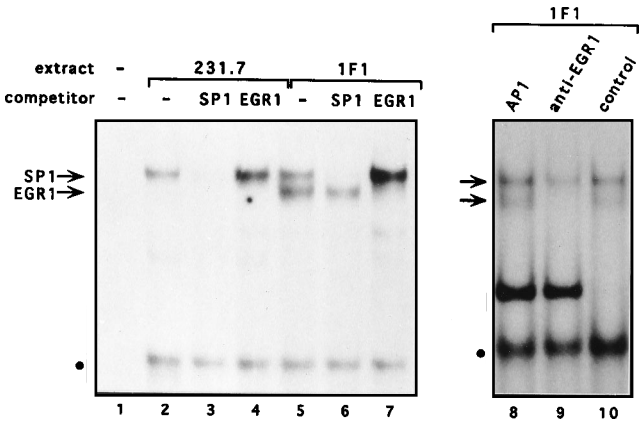

FIG. 7. EGR1 binds to a region at bp −301 within the human CD44 promoter. Five micrograms of nuclear extracts prepared from 231.7 (lanes 2 to 4) and 1F1 (lanes 5 to 10) cells which had been stimulated with 10 ng of PMA per ml for 2 h was tested for binding to a ^{32}P-labeled oligonucleotide probe spanning bp −288 through −307 of the CD44 promoter. Unlabeled oligonucleotides containing an Sp1 binding site (lanes 3 and 6), two EGR1 binding sites (lanes 4 and 7), an AP1 binding site (lane 8), and antisera specific for EGR1 (lane 9) or CDK4 (lane 10) were preincubated with the nuclear extracts for 15 min at 4°C prior to the addition of the probe. Binding reactions were carried out for 15 min at room temperature and were followed immediately by separation of binding reaction mixtures on either a 6% polyacrylamide–1× Tris-glycine gel (lanes 1 to 7) or a 4% polyacrylamide–0.5× TBE gel. Gels were dried and autoradiographed. The mobilities of EGR1- and SP1-specific binding complexes (based on competition experiments) are indicated on the left. •, nonspecific binding complex.

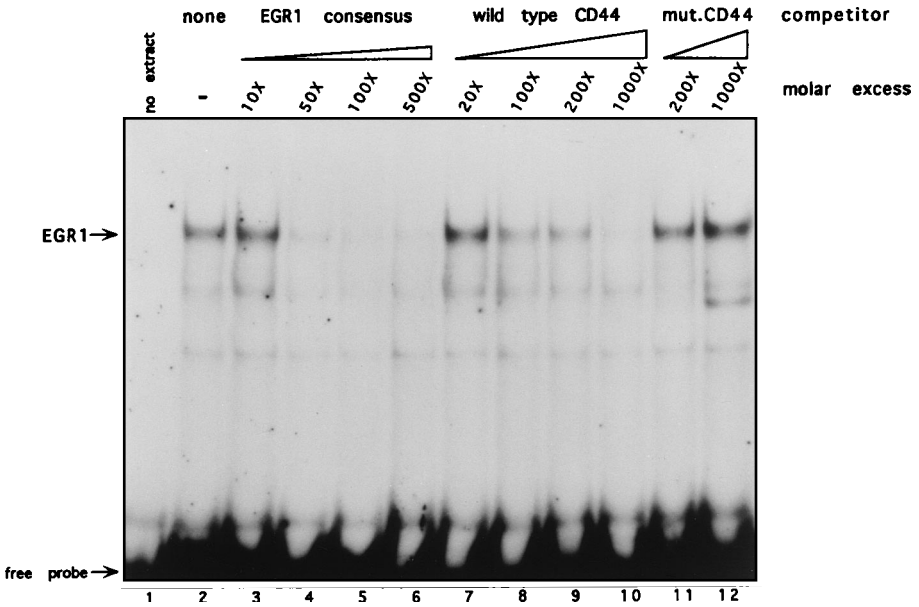

FIG. 8. The affinity of EGR1 for the nonconsensus motif at bp −301 of the CD44 promoter is lower than that for the consensus binding motif. EMSA were performed with 5 μg of nuclear extracts from 1F1 cells stimulated for 2 h with 10 ng of PMA per ml and a ^{32}P-labeled oligonucleotide probe containing two EGR1 consensus binding motifs. To assess the relative affinity of EGR1 to different binding motifs, unlabeled oligonucleotide competitors containing two EGR1 consensus motifs (lanes 3 to 6) or the nonconsensus binding motif at bp −301 were titrated into the binding reaction mixtures. The molar excess of unlabeled EGR1 binding sites for each competition is indicated above each lane. The competitor used in lanes 11 and 12 contains a 3-bp mutation in the bp −301 binding motif. Binding reaction mixtures were separated on a 4% polyacrylamide–0.5× TBE gel, and the dried gel was autoradiographed.

this motif is EGR1 (Fig. 3B), we inferred that this complex must be due to EGR1. To formally show that this complex was the result of EGR1 binding rather than that of another family member, we carried out antibody blocking experiments. Antibodies directed to the unique C terminus of this family member (15, 26, 44, 56) but not a control antiserum (against CDK-4) specifically blocked formation of the complex specific to 1F1 extracts (Fig. 5, compare lanes 9 and 10).

To determine the relative affinities of EGR1 for the bp −301 motif and the EGR1 consensus motif, nuclear extracts from 1F1 cells were again used as a source of cellular EGR1. The extracts were incubated with an oligonucleotide containing two tandem EGR1 binding motifs, and binding was inhibited with a titration of cold competitor oligonucleotides (Fig. 8). Addition of as little as a 50-fold molar excess of consensus EGR1 motifs resulted in a complete inhibition (Fig. 8, lanes 3 to 6). In contrast, a 200- to 1,000-fold molar excess of the nonconsensus (bp −301) binding site was necessary to achieve complete inhibition (Fig. 8, compare lanes 7 to 10 and lanes 3 to 6). The addition of an oligonucleotide containing a mutated EGR1 motif resulted in no competition, even at the higher concentration (Fig. 8, lanes 11 and 12). On the basis of the number of EGR1 binding sites needed to inhibit binding to the probe, EGR1 has an estimated 4- to 10-fold-higher binding affinity for the consensus than for the bp −301 nonconsensus motif.

Expression of EGR1 induces CD44 promoter activity. Determination that EGR1 binds to the bp −301 site on the CD44 gene provided a mechanism for the direct interaction of the EGR1 transcription factor and the CD44 promoter. To directly assess the role of EGR1 in the induced transcription of the CD44 gene, we performed transient cotransfection analysis with a CD44 promoter-CAT reporter fusion and a vector containing the murine *egr*-1 gene under the transcriptional control of the β-actin promoter. This analysis was begun by using a reporter construct containing both the bp −600 and −301 binding sites (although our studies discussed above indicate that the bp −600 site is unable to bind EGR1) and extending 3′ through the CD44 transcription initiation site (pSacBCAT) (57). Multiple cotransfections with an *egr*-1 expression vector and pSacBCAT in 231.7 cells gave a level of CAT activity which was below the sensitivity of our assay (data not shown). The lack of detectable expression was attributed to a combination of low intrinsic promoter activity coupled with, more importantly, the low transfection efficiency of murine B lymphocytes.

To enhance the level of basal transcription, the region spanning bp −701 through −151 was cloned upstream of the minimal herpes simplex virus thymidine kinase promoter to generate pBLCD44. The −701 to −151 region retains both the bp −600 and −301 regions as well as the two Sp1 binding sites and the AP1 site but does not include the putative endogenous CD44 transcription initiation sites (Fig. 9A). Because we were using a human promoter construct in murine cells, we tested the ability of both human EGR1 and murine EGR1 to transactivate the CD44 promoter. Cotransfection of pBLCD44 with an expression vector for the murine EGR1 (pAC-mEGR1) or the human EGR1 (pAC-hEGR1) resulted in 5- and 7-fold inductions of CAT activity, respectively, compared with that for the empty expression vector (Fig. 9B). In a subsequent experiment, mutation of the bp −301 site (see Fig. 10A) was shown to abolish the ability of EGR1 to transactivate the transfected CD44 promoter-CAT construct. In this experiment, transfection of the wild-type CD44-CAT reporter with pAC-hEGR1 resulted in a 2-fold induction of CAT activity in 231.7 cells, while the same reporter with a mutated bp −301 EGR1 binding site exhibited no induction (1.1-fold). Although inducible activity was relatively low in this experiment, the results clearly establish the necessity for the integrity of this site for EGR1 transactivation. These results, together with DNA binding data (Fig. 7), indicate that EGR1 binding to the CD44 promoter at bp −301 results in the induction of CD44 transcription.

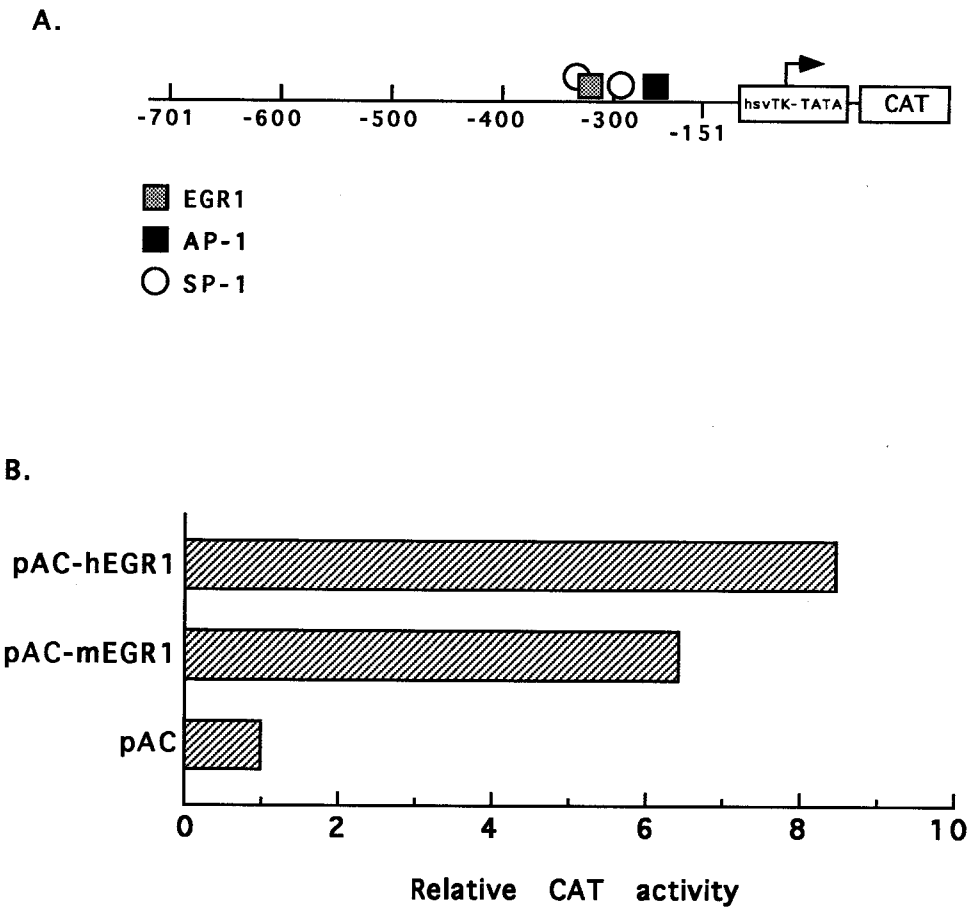

FIG. 9. EGR1 transactivates a CD44 reporter construct. (A) Schematic representation of CD44 promoter-CAT reporter construct. A 550-bp region of the CD44 promoter was cloned immediately 5′ of a minimal herpes simplex virus-thymidine kinase (hsvTK) promoter driving a CAT reporter gene. The EGR1 binding site is at bp −301. (B) 231.7 cells were transfected with 20 μg of pBLCD44 and 5 μg of expression vectors for either human EGR1 (pAC-hEGR1), murine EGR1 (pAC-mEGR1), or no insert (pAC). Ten micrograms of pSV_2PAP was included to control for transfection efficiency. Amounts of acetylated and nonacetylated chloramphenicol were measured from thin-layer chromatography plates by PhosphorImager analysis, and percent conversion was calculated as described in Materials and Methods. All values were corrected for transfection efficiency by using placental alkaline phosphatase activity.

The EGR1 binding motif at bp −301 of the CD44 promoter is necessary for full transcriptional induction. The importance of the EGR1 binding motif during stimulus-induced transcription of CD44 was assessed by introducing a 3-bp mutation into the bp −301 motif in pBLCD44 (Fig. 10A). The mutated motif retained the ability to bind Sp1 (data not shown) but no longer bound EGR1 (Fig. 8, lanes 11 and 12). The two CD44 reporters, pBLCD44 and the mutated pBLmCD44, were used in transient transfection experiments in primary, nontransformed B lymphocytes previously stimulated with LPS for 72 h to increase transfection efficiently (37). This system was necessary for these studies because stimulation of EGR1-expressing 1F1 cells results in cell cycle arrest and apoptosis at 24 h, contraindicating its use in a 24-h CAT assay. However, even more important, the use of LPS-pretreated cells allowed the investigation of EGR1 regulation of the CD44 promoter in a nontransformed B-cell population at physiological levels of endogenous EGR1 protein. Although basal levels of CD44 mRNA were higher in these cells than in unstimulated B lymphocytes, stimulation with either PMA or BCR cross-linking nonetheless induced increased message levels of CD44 (data not shown). CAT activity was increased (3.3-fold) when LPS-pretreated cells were transfected with pBLCD44 and stimulated with PMA for 24 h (Fig. 10B). In contrast, stimulation of pBLmCD44-transfected cells resulted in only minimal (1.1-fold) and insignificant ($P < 0.01$) induction. The presence of an intact EGR1 binding motif, therefore, was necessary for full transcriptional induction of this gene when physiological levels of EGR1 were mediating this response. Furthermore, the apparent complete abrogation of transcriptional induction by mutation of the EGR1 binding site argues that other potential transcription factors that may be induced by stimulation of these cells play insignificant roles in the regulation of this promoter under these conditions.

DISCUSSION

Stimulation of B lymphocytes through the BCR initiates a set of second-messenger pathways leading to the rapid and transient induction of immediate-early genes, many of which encode transcription factors (28, 39, 42). Through their ability to regulate expression of stimulus- and tissue-specific genes, the protein products of these genes are believed to function to translate these second-messenger pathways into the alterations of phenotype and activation state that accompany specific cellular responses (35). Identification of the target genes for proteins encoded by specific immediate-early genes is an approach to understanding how BCR-induced signals affect B-cell phys-

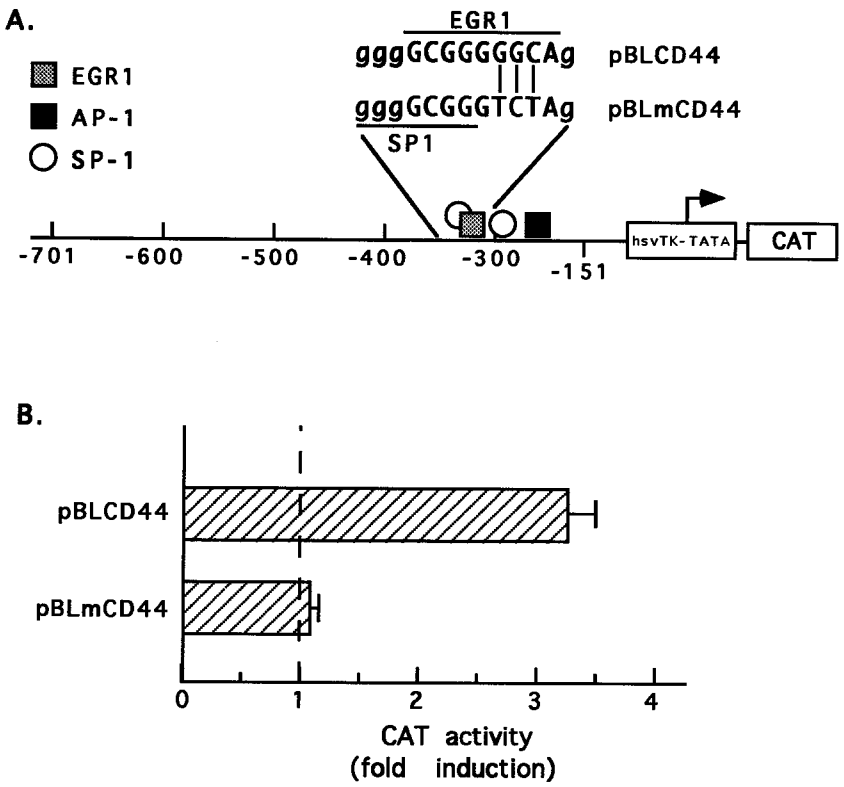

FIG. 10. The EGR1 binding motif at bp −301 is necessary for PMA-induced CD44 transcription in nontransformed lymphocytes. (A) Schematic representation of CD44 promoter constructs used in panel B. pBLCD44 and pBLmCD44 differ by the indicated 3-bp mutation which abolishes EGR1 binding. (B) Forty micrograms of pBLCD44 or the mutant reporter pBLmCD44 were transfected into 5×10^7 LPS-pretreated B lymphocytes as described in Materials and Methods. Twenty-four hours following transfection, the cultures were evenly divided and either stimulated with 10 ng of PMA per ml or left unstimulated. Following an additional 24 h of incubation, the cells were harvested and assayed for CAT activity. The results depict the mean fold induction (compared with unstimulated cells) from five independent experiments ± the standard error of the mean.

iology. We have identified the CD44 gene as a target of EGR1 regulation in antigen receptor-stimulated B lymphocytes. This finding provides insight into a key element, allowing us to trace BCR-initiated signaling pathways to a defined B-cell phenotypic change that is relevant to its participation in an immune response.

Our evidence for EGR1 regulation of CD44 expression is based upon an experimental system which allows comparisons of gene regulation in subclones of a single B-cell line. To the extent that we have been able to evaluate, these subclones differ only in their ability to express *egr*-1. Using these subclones, we have shown that expression of EGR1 correlates with the ability of BCR signaling to promote induction of transcription from the endogenous CD44 gene. In addition, nuclear extracts from cells containing EGR1, but not those from cells deficient in EGR1, have a binding activity with an identified EGR1 binding motif in the CD44 promoter. Transient transfection assays with a CD44 promoter-CAT reporter construct definitively show that EGR1 upregulates transcription of the CD44 gene and that the EGR1 binding motif located at bp −301 is necessary for full induction in primary, nontransformed B-cell blasts.

The transient transfections described in this report were carried out by using a chimeric reporter in which the 5′ flanking region of the CD44 gene regulated transcription from a minimal promoter containing the heterologous herpes simplex virus-thymidine kinase TATA box as a transcription start site. Multiple transfection experiments using a reporter (pSacBCAT) which uses the CD44 transcription start site resulted in no detectable CAT activity with either 231.7 or LPS-pretreated B lymphocytes even though this promoter is active in murine NIH 3T3 cells (33a). We believe that a combination of the low transfection efficiency of B lymphocytes and the low promoter activity results in transcriptional activity below the level of detection with our assay. Chimeric reporters with promoter fragments driving heterologous promoters are commonly used to investigate gene regulation. We cannot rule out the possibility that regulation of the chimeric pBLCD44 reporter is different from either that of pSacBCAT or that of the endogenous CD44 gene; however, the data obtained from transient transfections of pBLCD44 complement our studies with the WEHI-231 subclones in which regulation is mediated through the normal CD44 promoter in its relevant chromosomal context. In the WEHI-231 system, PMA-induced transcription and anti-μ antibody-induced transcription from the endogenous CD44 promoter are dependent on the presence of EGR1. In cells that lack EGR1 (231.7), a minimal increase in transcription was observed following stimulation. Similarly, transcriptional induction of the promoter construct was dependent on the presence of an intact EGR1 binding site (pBLCD44 versus pBLmCD44, Fig. 10). Thus, anti-μ antibody-induced transcription and PMA-induced transcription of CD44 require an interaction between EGR1 and the EGR1 binding site at bp −301 of the promoter.

Identification of EGR1 target genes has been limited to date, resulting in the identification of a small number of largely, but not exclusively, tissue-specific genes (1, 20, 33, 46). Many of these EGR1-regulated genes are rich in G+C content and lack

the TATA and CCAAT elements classically found in eukaryotic promoters. Similarly, the human CD44 promoter lacks both TATA and CCAAT elements, and the region between bp +1 and −701 (investigated in this report) contains approximately 65% G+C (57). The promoter contains three Sp1 sites and two EGR1-like motifs within 1 kb of the start site; EGR1 binds detectably only to the bp −301 site.

The 9-bp consensus binding motif for EGR1 was originally identified mainly on the basis of experiments using bacterially expressed or in vitro-translated EGR1 protein, often not utilizing the entire coding sequence (7, 12, 48). More recently, a systematic PCR-based approach was taken to redefine and extend these studies (62). While this study indicated that there is more variability in the binding sequence than previously appreciated and that flanking sequences play a role in binding site affinity, it also utilized bacterially expressed proteins. One previous study has addressed the DNA binding specificity of cellular EGR1 (7) and showed low-affinity binding of a cellular factor to the nonconsensus motif found at bp −279 of the *egr*-1 promoter (which is identical to the nonamer at bp −301 of the CD44 promoter). This factor was not conclusively shown to be EGR1 and may have been a related family member. We have demonstrated binding of mammalian EGR1 to the nonconsensus motif located at bp −301 by using a combination of nuclear extracts that differ in respect to EGR1 and antibody blocking. In addition, consistent with findings obtained with synthetic EGR1 protein (6), the affinity of EGR1 for this motif is lower than that for the consensus EGR1 binding motif (Fig. 8B).

The low affinity of EGR1 for this site may affect competition between EGR1 and Sp1 for binding to this motif. Overlapping EGR1-Sp1 binding motifs are common in EGR1-regulated genes, and competition of these factors for the site plays a role in regulation of some of these (1, 16). We have not formally addressed competition between these two factors for this motif. The Sp1 sites were shown to be functionally significant in expression of this gene in neuroblastoma cells (57). While data do not support a role for Sp1 in the inducible expression of CD44, it is possible that Sp1 is important for basal expression of CD44. Consistent with this possibility are the observations that Sp1 is constitutively expressed in nearly all cell types (14, 52) and that previous studies implicating a role for Sp1 in CD44 regulation (57) have only evaluated noninducible expression.

Previous studies of the transcriptional regulation of CD44 in cloned embryo rat fibroblast cells have implicated the AP1 site in *ras*-dependent induction of CD44 (24). Although our studies do not specifically address the importance of this site in B lymphocytes, we have found that there is little if any stimulation-dependent increase in CAT activity from pBLCD44 in the absence of EGR-1 binding in primary B cells (Fig. 10B). This does not rule out a role for AP1 in B-lymphocyte regulation of CD44. It is possible that AP1 and EGR1 are both necessary for optimal transcription and that the lack of either transcription factor results in a reduction in transcription. It should be noted here that we do not consistently observe a difference in CD44 inducibility in anti-μ antibody-stimulated splenic B cells from normal and EGR1-deficient mice (29). However, given that *egr*-1 is a ubiquitously expressed transcription factor and given its diverse role in cellular proliferation and differentiation, it is probable that other family members that bind to the same sites and are similarly ubiquitously expressed, such as *egr*-2 and *egr*-3, are likely to compensate for a loss in *egr*-1 expression (29). Importantly, *egr*-2 and *egr*-3 are expressed in murine splenic B cells (Fig. 2) (unpublished data). For this reason, in the case of transcription factor genes belonging to extended families, deletion studies with whole animals targeting single family members that affect multiple and likely essential developmental and physiological processes are unlikely to be definitive, given the enormous selective pressure for compensation to ensure viable offspring. In the specific case discussed here, perhaps a better approach would be to use the recombinase gene-defective mouse system so that the targeted gene could be restricted to cells of the T and B lineages. However, even potential effects on cell development could complicate these studies.

CD44 has been hypothesized to play a role in both physiological and pathologic cellular migration and trafficking. Several tumor models have implicated splice variants of CD44 in increased metastatic potential (24, 50). In nontransformed lymphocytes, CD44 has been implicated in lymphocyte extravasation into secondary lymphoid tissue (58). In vitro, cell adhesion models have demonstrated that CD44 is the primary cell surface receptor for HA (2). In vivo stimulation of B lymphocytes leads to the increased expression of a number of cell surface proteins, including CD44, that are involved in lymphocyte homing and migration. CD44 protein levels are increased within 24 h following stimulation through the BCR on primary cells (5). However, the relationship of B-lymphocyte CD44 surface levels and the ability to bind HA is complex. Differences in N glycosylation of newly synthesized CD44 have been implicated in the ability to bind HA (22, 27, 32). While a signal through the BCR complex is sufficient to increase the density of surface CD44 on B lymphocytes, affinity for HA does not increase in the absence of additional signals such as the presence of interleukin-5 (15, 22, 26, 47, 60). Activated B lymphocytes, with high levels of CD44 transcription, could receive this additional signal upon entering the appropriate secondary lymphoid tissue and begin expressing modified (HA-binding) CD44. Both increased transcription of the gene (the result of BCR cross-linking) and an alteration in the posttranslational milieu of the cell (due to an interleukin-5 signal) would be necessary to allow increased HA binding and retention in the lymphoid tissue.

In summary, the results presented in this paper offer evidence of a linkage between BCR-induced biochemical changes and downstream alterations in B-lymphocyte phenotype. It is likely that EGR1 participates in the transcriptional regulation of numerous other genes involved in the B-lymphocyte activation response. It is perhaps interesting that we have recently identified another EGR1 target gene in BCR-stimulated B cells (34). This gene, *Icam*-1, like the CD44 gene, encodes an adhesion molecule involved in the B-cell-mediated immune response. This suggests the possibility that EGR1 is involved in the coordinate regulation of cellular interaction proteins involved in early phases of the humoral immune response.

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