Epidermal Growth Factor Induces CD44 Gene Expression through a Novel Regulatory Element in Mouse Fibroblasts*

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Growth factors coordinately regulate a variety of genes associated with pathological states including tumor invasion and metastasis. Overexpressed epidermal growth factor receptor (EGFR) on tumor cell surfaces is associated with enhanced cell attachment and migration into extracellular matrices, which promotes tumor aggressiveness. We have demonstrated that epidermal growth factor (EGF) up-regulates the cell surface adhesion molecule CD44 at both the mRNA and protein levels on mouse fibroblasts expressing full-length wild-type EGF (NR6-WT) but not on EGFR-deficient cells (NR6-P). This increases cell attachment to hyaluronic acid. In this investigation, transcriptional regulation of CD44 by EGF was confirmed by defining an EGF-regulatory element. By employing human CD44 gene promoter-chloramphenicol acetyltransferase (CAT) constructs transfected into NR6-WT cells, EGF inducibility was observed within a 120-base pair (bp) DNA fragment located 450 bp upstream of the RNA initiation site. Differential EGF inducibility was found among different cell lines chosen, indicating a 3.2- and 1.8-fold enhancement in DU145 cells carrying exogenous wild-type EGFR and in MCF-7 cells, respectively, while minimal EGF induction was found in cervical cancer HeLa cells. Utilizing gel shift assays, a time-dependent increase of DNA-protein complex formation was found upon EGF stimulation in NR6-WT cells but not in NR6-P cells. Based on these observations, a novel 22-bp EGF regulatory element (ERE) (5'−944CCCTCTCTCCAGCTCCTCTCCC−3') was isolated from the CD44 gene promoter. This ERE conferred DNA-protein binding ability in vitro, as well as the full functional recovery of EGF inducibility of CAT activity when linked to a homologous CD44 promoter or a SV40 promoter driving a CAT reporter gene. A two-base mutation of the ERE completely eliminated its binding activity as well as its EGF inducibility of CAT expression. Our studies indicate that EGF induces CD44 gene expression through an interaction between a specific ERE and putative novel transcriptional factor so as to regulate cell attachment to extracellular matrix.

Growth factors in the extracellular milieu can convey signals to cells via transmembrane surface receptors to regulate a variety of downstream events. Activation of the epidermal growth factor receptor (EGFR1) upon binding of its ligands (EGF, transforming growth factor-α, HB-EGF, and amphiregulin) under both normal and pathological conditions, initiates a cellular cascade which results in major pleiotropic changes in many cell types (1, 2). Aberrant overexpression of EGFR on tumor cells is associated with increased proliferation, enhanced cell attachment, and migration into extracellular matrices, progression to a transformed phenotype and in vivo aggressiveness (3–7). Among the regulated genes are those encoding cell surface adhesion molecules (8–10) as well as nuclear transcriptional factors that regulate gene expression. Cell surface glycoprotein CD44, an adhesion molecule that binds to the extracellular matrix components, hyaluronic acid (11) and osteopontin (12), has been postulated to modulate tumor invasion and metastasis (13–15). Our previous studies with mouse 3T3 fibroblasts demonstrated that EGF induced CD44 gene expression on both the protein and mRNA levels and that this was accompanied by enhanced cell attachment to the extracellular matrix component hyaluronic acid (16).

Although it is known that EGF regulates gene transcription of the early responsive genes, c-myc, c-fos, and c-jun, through signaling mechanisms that are shared with the protein kinase C signaling pathway (17–22), few EGF regulatory elements or their corresponding DNA-binding proteins have been identified. A serum-response element mediates EGF-stimulated c-fos transcription (23, 24). Sp1 is required by EGF to stimulate the human gastrin gene upon binding to a 16-bp overlapping GC-rich EGF response element (25), and elements in the prolactin and tyrosine hydroxylase promoter can serve as an EGF response element (ERE) (19, 23, 24, 26). However, these sequences do not connote a specific ERE.

Structural analysis of the CD44 upstream regulatory region in human neuroblastoma cells (27) revealed that there were neither typical TATA nor CCAAT boxes in the promoter region, although approximately 150 bp adjacent to the 5'-end of the gene itself was sufficient to induce substantial basal transcription of the chloramphenicol acetyltransferase (CAT) gene. Despite the existence of three consensus hexanucleotide GGCGGG sequences, Sp1 binding to the CD44 gene upstream region has not been demonstrated. Rather, the cis-regulatory element, which was known to contribute to the down-regulation of CD44 in neuroblastoma cells, was identified as being a 120-bp DNA fragment located 450 bp upstream of the RNA initiation site. To gain further insight into the molecular mechanism by which EGF regulates CD44 gene expression, we investigated transcriptional regulation of CD44 gene by EGF using the bacterial CAT reporter system. Both in vitro DNA-
protein binding ability and in vivo EGF inducibility were examined in both wild-type as well as mutational EREs. Here, we report the existence of a transferable 22-nucleotide sequence which confers EGF responsiveness to transcriptional units.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and EGF Induction—**Parental murine NR6 cells (P), which are 3T3 derivatives devoid of endogenous EGF (28), were stably transduced by retrovirus-mediated infection with holo (wild-type) EGFR (29). These cell lines have been previously characterized, and are maintained at 37 °C in a 5% CO2 environment, in minimal essential medium (Life Technologies, Inc.) supplemented with 7.5% fetal bovine serum, 100 ng/ml plasmid DNA containing CD44 cDNA, 3 mM butyryl coenzyme A, and sodium pyruvate with 350 μg/ml G418 as a selective marker for the NR6-WT cells. Cells were carefully passaged before they reached 90% confluence to eliminate spontaneous morphological change. EGF induction was accomplished with 10 ng EGF (Life Technologies, Inc.) in complete medium containing 0.1% dialyzed fetal bovine serum for different time periods. Other cell lines used were: DU145 carrying exogenous wild-type EGFR (31) in Dubecco’s modified Eagle’s medium/F-12 media supplemented with 100 μg/ml penicillin and streptomycin, 7.5% fetal bovine serum, and 1 mg/ml G418 as a selective marker for the MCF-7 WT cells. Each of these cell lines were maintained according to recommendations from the ATCC.

**Analysis of Newly Synthesized CD44 mRNA in Nuclear Run-on Assays—**Preparation of nuclei and RNA labeling followed standard protocols (30). Briefly, 1 × 10⁶ NR6-P or NR6-WT cells were treated with 10 ng EGF for 0, 1, 3, 6, or 12 h, washed with ice-cold phosphate-buffered saline, and preincubated in cold lysis buffer (10 mM Tris-Cl (pH 7.4), 3 mM CaCl₂, and 2 mM MgCl₂). The cells were resuspended in 2 ml of lysis buffer followed by further lysis in 2 ml of Nonidet P-40 buffer (addition of 1% Nonidet P-40 to the lysis buffer) before disruption by 10 strokes in a cold Dounce homogenizer. Disruption was confirmed by light microscopic evaluation after trypan blue staining. Nuclei were pelleted by centrifugation at 500 × g, 4 °C for 5 min, and then resuspended in ice-cold glycerol storage buffer (50 mM Tris-Cl (pH 8.0), 0.4% glycerol, 5 mM MgCl₂, and 0.1 mM EDTA) followed by immediate return to dry ice before being stored at −70 °C for later use.

Enzyme assay systems were used to determine the in vivo and in vitro EGF inducibility were examined using newly synthesized CD44 RNA as a probe. Briefly, 1 × 10⁷ cells were lysed, and nuclei were isolated from 0.1% dialyzed fetal bovine serum. Cells were then treated with or without 10 ng/ml EGF for 24 h, and then treated with or without 10 ng/ml EGF for 24 h, and then treated with or without 10 ng/ml EGF for 24 h, and then treated with or without 10 ng/ml EGF for 24 h, and then treated with or without 10 ng/ml EGF for 24 h. Supernatants were further purified by heparin-affinity chromatography (Bio-Rad) and eluted from a concentration gradient of 50 to 350 mM KCl. Eluates were fractionated into 12 fractions each of 6.7 ml, and each was assayed for CAT activity by measuring the amount of radiolabeled 3-5’-cyclic adenosine monophosphate by [γ-32P]-5’-ATP incorporation into the DNA probe at 1 or 100 ng, and unlabeled pRBV CAT insert DNA at 1 or 10 ng were preincubated with the nuclear extracts at 25 °C for 30 min before being placed in reaction with the labeled probe which serves as the competitor control. Unlabeled synthetic oligonucleotides were 5’-end-labeled with γ[32P]-ATP. 2 × 10⁴ cpm of each probe was applied to 20 μl of premixed gel shift reaction containing 25 mM Tris-Cl (pH 8.0), 6.25 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, and 40 ng of probe DNA in the presence of 4 μg of nuclear extract at 25 °C for 1 h. DNA was separated on a 4% polyacrylamide gel at 75 V for 3 h, dried, and visualized by autoradiography. The DNA probe was then excised from the gel and eluted from the gel in buffer containing 1× MDE buffer. The eluted DNA was then digested with HindIII and XbaI, and the resulting DNA fragments were purified on a 3% agarose gel. The purified DNA was incubated with 5 μg of poly(dI-dC) in the presence of 4 μg of nuclear extract at 25 °C for 1 h. DNA was separated on a 4% polyacrylamide gel at 75 V for 3 h, dried, and visualized by autoradiography. The DNA probe was then excised from the gel and eluted from the gel in buffer containing 1× MDE buffer. The eluted DNA was then digested with HindIII and XbaI, and the resulting DNA fragments were purified on a 3% agarose gel. The purified DNA was incubated with 5 μg of poly(dI-dC) in the presence of 4 μg of nuclear extract at 25 °C for 1 h.
denatured polyacrylamide gel containing 45 mM Tris base, 45 mM boric acid, and 2.5% glycerol under nondenaturing conditions. After exposure to x-ray film, gel shift bands were quantified by densitometry (Image 1.47 software).

**DNase I Protection Footprinting Assays**—DNase I protection assays were performed in 50 μl of reaction with 0.5, 2, or 4 μg of nuclear extract from NR6-WT cells treated with or without 10 nM EGF for 6 h and 2 × 10⁹ cpm of the 120-bp [α-32P]dCTP-labeled DNA probe as described in the gel shift assay. The nuclear extract was preincubated without probe for 10 min on ice in the same buffer as used in the gel shift assay, and incubated for another 30 min on ice after the addition of probe. Digestion was initiated with the addition of 1 unit of DNase I (Promega) in 25 mM CaCl₂ at room temperature and terminated after 1 min with 90 mM EGF for 0, 1, 3, 6, or 12 h were labeled with [α-32P]dUTP and hybridized individually to a nitrocellulose membrane dot blotted with 5 μg of denatured and linearized plasmid containing CD44 cDNA, α-actinin, or pBluescript II SK+ vector control (A). The CD44 transcriptions from both cell lines, at each time point, were equalized by comparison with the α-actinin transcripts (B).

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**EGF Up-regulates CD44 Gene Transcription**—Our previous data indicated that EGF up-regulated cell attachment of NR6 mouse fibroblastic cells to hyaluronic acid by increasing the expression of the cell surface adhesion molecule CD44 at both the protein and mRNA levels (16). EGF treatment of NR6 cells expressing wild-type EGFR resulted in a 3-4-fold up-regulation of CD44 protein expression with a maximum at 24 h, as well as a 3-fold increase of mRNA levels after 6-24 h of EGF treatment (16). The CD44 mRNA in NR6 cells were determined by nuclear run-on analysis to confirm the transcriptional regulatory mechanism (Fig. 1). Both NR6 parental cells without endogenous EGFR (−P) and NR6 cells with transfected EGFR (−WT) were preincubated with 10 nM EGF for various times before collecting and labeling new transcripts. In agreement with earlier Northern blot analysis, there was no perceptible change in the rate of CD44 transcription with NR6-P cells, while a 3-fold induction was observed maximally at 6 h in NR6-WT cells in the presence of EGF. This was seen to decline by a third at 12 h, identical to the transient wave of mRNA seen on Northern blot analysis (data not shown). These results support the contention that EGF regulates CD44 mRNA levels by increasing transcription in a time-dependent manner at least during short-term EGF exposure.

**Functional Characterization of the EGF Regulatory Element (ERE) in the CD44 Gene**—To identify the sequence that mediates EGF responsiveness in the CD44 gene, a series of DNA fragments containing the sequence of the human CD44 gene upstream regulatory region (pRBCAT) and restriction enzymatic internal truncations (pSacBCAT and pRVBCAT) and deletions of the human CD44 gene upstream region (pRB-SRCAT) were obtained and placed into a bacterial CAT reporter gene (Fig. 2A). CAT activities were measured on electroporated transfected NR6-WT cells in the presence or absence of EGF. Consistent with the previous finding in human neuroblastoma cells (26), all constructs that contained the 150-bp sequence upstream of the transcription initiation site conferred substantial basal CAT activity on the constructs (Fig. 2B) when compared with the promoterless CAT plasmid.

The observation that not only did pSacBCAT elicit a 2-fold higher basal CAT activity than pRVBCAT or pRB-SRCAT, both of which were lacking a 120-bp fragment between SacI and EcoR sites (Sac-RV fragment), but that the same order of magnitude change was seen in comparison to pRBCAT, which contains this fragment, suggested that a negative cis-element upstream of the SacI site and a stimulatory cis-element on the Sac-RV fragment may contribute to the basal promoter activity. Moreover, as there was an increase in EGF-mediated induction of CAT activity in pRBCAT and pSacBCAT (2.5- and 2.3-fold, respectively) (Fig. 2C), the only construct with a Sac-RV fragment sequence, this was strong presumptive evidence that an ERE was present in this region.

**Differential EGF Induction of CAT Gene Transcription between Different Cells**—To test whether the observed relatively low EGF induction in NR6 cells (2.3-fold) was due to low promoter activity or is the result of a cell-specific effect, three transformed human cell lines, which express EGFR and CD44 and are derived from prostate, DU145-WT, breast, MCF-7, or cervix HeLa, were employed in our transfection/CAT system (Fig. 3). All cells were transfected by pCAT, pRVBCAT, and pSacBCAT constructs via standard electroporation methods. Basal level of CAT activity was determined by cotransfection of pCAT and pRB-SRCAT (2.5- and 2.3-fold, respectively) (Fig. 2B) when compared with the promoterless CAT plasmid. CAT activities were measured on electroporated transfected NR6-WT cells in the presence or absence of EGF. Consistent with the previous finding in human neuroblastoma cells (26), all constructs that contained the 150-bp sequence upstream of the transcription initiation site conferred substantial basal CAT activity on the constructs (Fig. 2B) when compared with the promoterless CAT plasmid.
tions of the assay. Moreover, additional experiments demonstrated that CAT induction of NR6-WT cells began at a low dose of 5 nM EGF and was sustained at an EGF concentration of up to 20 nM (data not shown), indicating no EGF dose-dependent bidirectional effect.

**A EGF Inducible Nuclear Protein Binds to the Sac-RV Fragment of the CD44 Promoter—**The presence of an EGF inducible DNA binding activity was sought by an electrophoretic mobility shift assay using the 32P-labeled Sac-RV DNA fragment (which contained the putative ERE described in the functional analysis), and nuclear extracts from NR6-P and -WT cells. Pilot experiments indicated that nuclear extracts eluted from heparin-affinity columns with 0.4M KCl buffer contained the DNA-protein complexes of interest. Binding specificity was assessed by examining the effect of excess amounts of poly(dI-dC) as nonspecific DNA, unlabeled Sac-RV fragment as specific competitor, and probe containing the CD44 promoter sequence from the pRVBCAT plasmid as random nonspecific DNA (Fig. 4). It was noted that three bands (labeled 2, 3, and 4) were eliminated by preincubating the labeled probe with 0.5 μg of poly(dI-dC), while the major band (labeled 1) retained significant binding, even in the presence of excess poly(dI-dC) (Fig. 4A). Furthermore, the major band could be competed only with increasing amounts of specific unlabeled Sac-RV probe (10–100 ng, equivalent to 20–200-fold excess of the labeled probe) (Fig. 4B) but not to excessive nonspecific probe derived from pRVBCAT (Fig. 4C).

The time course of EGF induction of this ERE response was assessed using nuclear extracts from both NR6-P and -WT cells, treated by EGF for 1, 3, 6, or 12 h (Fig. 5). Extracts were normalized to cell number with the loading amount ranging from 3.8 to 4.0 μg; 0.5 μg of poly(dI-dC) (500-fold excess) was also included in the reaction mixture to minimize nonspecific
binding. No change was observed in protein-DNA complex formation upon EGF stimulation in the NR6-P cells. However, in NR6-WT cells, the induction became evident (2-fold increase, n = 3), 3–6 h after exposure to EGF, and persisted for at least 12 h (Fig. 5B). It was noted also that this time-dependent induction of protein-DNA complex formation paralleled the mRNA wave demonstrated in both Northern blot and nuclear run-on assays, suggesting that the induction response required functional EGFR expressed on cell surfaces and that the Sac-RV fragment contained an ERE.

Identification of an ERE in the CD44 Gene Promoter—This work strengthened the hypothesis that EGF up-regulates CD44 gene expression through induction of transcriptional factor(s) binding to an ERE in the gene promoter region. To determine the specific sequence to which the transcriptional factor bound, we performed DNase I footprinting by using a nuclear extract from NR6-WT cells induced by EGF for 6 h and [α-32P]dCTP 5'-end labeled Sac-RV as the DNA probe. Two “footprints” were observed when 0.5 μg of poly(dI-dC), as nonspecific DNA competitor, was included in the binding reaction: footprint I over the sequence −5′-CTC-CTCTCC-3′ and footprint II over the sequence −604CTCCTCCTCC-503 (Fig. 6). Both sites were better protected from DNase I digestion when an increasing amount of nuclear extract (0.5, 2.0, and 4.0 μg in lanes 2, 3, and 4, respectively, from non-EGF treated cells; lanes 5, 6, and 7, respectively, from EGF-treated cells) was added. The specificity of the protein binding was also demonstrated by competition with excess poly(dI-dC), as nonspecific DNA, which abolished a putative third footprint and with unlabeled Sac-RV fragment, as specific probe, which competed away all footprints while the RVB fragment, acting as an unlabeled random nonspecific competitor, did not affect the DNase I protection (data not shown). It should be noted that both footprints are partially palindromic in themselves and are inverse repeats of each other, and that the 2-base nucleotides between them were partially protected.

A sequence search in GenBank against known transcriptional factors did not reveal any significant homology to the two separate footprints, either alone or together. Therefore, this sequence from −583 to −604 on the CD44 gene upstream regulatory region appears to be a novel EGF-regulatory element in mouse fibroblast NR6 cells.

EGF-induced Nuclear Protein Specifically Binds to the ERE—The sequence-specific binding of the NR6 cell nuclear extract was further studied in a gel shift assay by using the 32P-5'-end-labeled Sac-RV fragment and an unlabeled 22-bp double-strand synthetic oligonucleotide as competitor: 5'-CCCTCCTCCTCCAGCTCCTCCTCC-. The protein-DNA complex formation recognized as the EGF-inducible nuclear protein was noted to decrease as an increasing amount of unlabeled synthetic oligonucleotides (1–10 pmol, equivalent to 10- and 100-fold excess of labeled probe, respectively) was applied to the gel shift reaction mixture (Fig. 7B). This suggested that this synthetic sequence could competitively bind the same EGF-induced protein.

To confirm the binding specificity of this ERE, the Sac-RV fragment (probe 1), the 22-bp oligonucleotide sequence (probe 2), and two oligonucleotides containing either the 5' or 3' half of the palindromic structure (5'-CCCTCCTCCTCCAG- and 5'-CAGCTCCTCCTCCC-) (probes 3 and 4, respectively) were 5'-end-labeled with [γ-32P]ATP followed by incubation with the NR6-WT nuclear extract and a gel shift assay was performed. As shown in Fig. 7A, only the 22-bp ERE (probe 2), but not the half-sequences (probe 3 and 4), was capable of binding the band
EGF Induces CD44 Expression

Fig. 7. EGF-induced protein-ERE complex formation in a gel shift assay. The DNA-protein complex formation was measured by using a 32P-labeled double SacB strand fragment (probe 1), 22-bp ERE (probe 2), 12-bp first half of ERE (probe 3), and 12-bp second half of ERE (probe 4), and by using nuclear extracts from NR6-WT cells as indicated. A, DNA-protein complex formation of each probe, with or without nuclear extracts, from the NR6-WT cells as indicated. B, ERE (probe 2) competition in protein-DNA complex formation. 1 and 10 pmol of annealed double-strand ERE were incubated with a nuclear extract from NR6-WT cells before adding labeled SacB fragment. C, EGF induced protein-ERE binding in the gel shift assay. Nuclear extracts from NR6-WT cells, with or without EGF treatment, for different time periods as indicated were incubated with labeled double probe 1 or probe 2 in a gel shift reaction. Lanes 1 and 2, probe 1 only, probe 1 with nuclear extract, respectively. Lanes 3–8, probe 1 was incubated with nuclear extracts from NR6-WT cells treated with 10 ng/ml EGF for 0, 1, 3, 6, and 12 h, respectively.

compatible with the Sac-RV probe, indicating that EGF-induced protein-DNA binding requires a full-length sequence.

The time-dependent EGF responsiveness was further demonstrated by using nuclear extracts from EGF-treated NR6-WT cells and labeled ERE (Fig. 7C). EGF inducibility was evident from 3 to 6 h after EGF stimulation, similar to the findings using the labeled Sac-Rv probe. Together, these results further strengthen the conclusion that the 22-bp sequence is an EGF-regulatory element which regulates CD44 gene expression.

ERE Is Necessary for EGF-induced Transcription—We demonstrated that the ERE sequence within the CD44 gene promoter is critical for both EGF-induced DNA-protein complex formation and EGF-induced transcription of the CAT gene. The identity of the ERE in the context of EGF induction of CD44 gene transcription was further assessed in a homologous and a heterologous CAT reporter system. Multiple EREs were placed in front of the CD44 gene promoter in the pRVBCAT construct, which lacked EGF inducibility of CAT gene transcription as described above. They were p1ERE.RVBCAT, p2ERE.RVBCAT, and p3ERE.RVBCAT, containing 1, 2, and 3 EREs, respectively. pRVBCAT and pSacBCAT served as the negative and positive controls. After transfection of NR6-WT cells with the various constructs, half the cell groups were stimulated with 10 ng/ml EGF for 24 h and compared in the CAT assay to the untreated matched control for increase of CAT activity secondary to EGF induction. All ERE-containing constructs elicit similar significant induction of CAT activity by EGF (2.8–2.4- and 2.5-fold for p1ERE.RVBCAT, p2ERE.RVBCAT, and p3ERE.RVBCAT, respectively), similar to the positive control (2.3-fold) (Fig. 8A). The fact that p1ERE.RVBCAT yielded at least equal EGF induction may imply that accessibility of the ERE because of its relative shorter sequence may be important in this process, although other mechanisms may also account for the observed changes.

To rule out the possibility that the CAT induction by EGF was the result of transcription initiated from the transcription start site of the transfectected CD44-CAT reporter gene, the ERE sequence was introduced into a heterologous SV40 promoter-driven CAT reporter system in both a sense and antisense orientation (pERE(s)SV40CAT and antisense orientation (pERE(α)SV40CAT), were transfected into NR6-WT cells for EGF induction of CAT activity. Data from four independent experiments of duplicates (*, p < 0.01).

Fig. 8. Functional analysis of ERE in homologous and heterologous CAT system. NR6-WT cells were transfected with various CAT constructs containing ERE as described before. Fold increase was measured as EGF induction from EGF-treated cells compared with untreated cells. A, homologous ERE constructs in EGF-irresponsive pRVBCAT vector containing 1, 2, and 3 ERE as p1ERE.RVBCAT, p2ERE.RVBCAT, and 3ERE.RVBCAT, respectively, as well as pCAT, pRVBCAT, pSacBCAT controls, were transfected into NR6-WT cells for EGF induction of CAT activity. B, ERE constructs in heterologous SV40-driven CAT vector (pSV40CAT) with both sense orientation (pERE(s)SV40CAT) and antisense orientation (pERE(α)SV40CAT), were transfected into NR6-WT cells for EGF induction of CAT activity.

The Integrity of the 22-bp ERE of CD44 Promoter Is Necessary for Full Transcriptional Induction—EGF-induced CD44 transcription is initiated by a specific nuclear protein binding to the ERE sequence of the CD44 gene promoter. We performed an experiment to test the specificity of the ERE sequence in the EGF induction event by point mutating the "T" nucleotides to two "C" (Fig. 9C), to disturb the potential binding motif (palindromic structure). The binding activity to the nuclear protein was measured in a gel shift assay. EGF inducibility of CAT transcription was also verified in a pSV40CAT reporter system by placing the mutated ERE in front of the SV40 promoter. In this analysis, the specific DNA-complex formation was observed to be enhanced when an unmutated ERE (WT) and an increasing amount of nuclear extract from EGF-treated NR6-WT were placed in the gel shift reaction mixture. Minor bands were, however, noted above the specific bands and were thought to be the result of nonspecific binding since no increasing binding effect was found when increasing amounts of nuclear extract was applied. Poly(dIdC), a nonspecific competitor, was able to eliminate these bands without affecting the major specific DNA-protein complex formation. In contrast, no significant DNA-protein complex formation was detected using the mutated ERE even in the presence of an excess amount of
EGF Induces CD44 Expression

were treated with or without 10 nM EGF for 24 h. Fold increase of CAT cells and fractionated on a native 4% nondenatured polyacrylamide gel constructs pSV-40 CAT, pERE(s)SV-40 CAT, and pERE(Mu)SV-40 CAT and response elements found in genes encoding c-fos, c-jun, and other genes whose protein products include gastrin (31), prolactin, tyrosine hydroxylase (19, 23, 25, 26), transin (20), and pS2 (21). These studies have shown that EGF stimulates transcription of genes through different cis-regulatory sequences. The ERE of c-fos is mediated by a serum response element or different factors which bind to unrelated cis-regulatory sequences needs to be addressed. Like the serum response element-mediated c-fos transcription, our ERE binds to nuclear proteins from EGFR-devoid NR6-P cells and unstimulated NR6-WT cells so to activate basal transcription in the context of the CD44 promoter when linked to CAT reporter gene. The specific DNA-protein complex formation derived from NR6-WT cells increased after EGF stimulation in a time-dependent manner. This implies that stimulation of CD44 gene transcription by EGF results from either an increased expression or increased activation of this cis-acting DNA-binding protein.

In summary, we conclude that EGF transcriptionally up-regulates CD44 gene expression. The 22-bp ERE from the CD44 gene promoter region is orientation-specific, and is necessary to enable EGF-induced CD44 gene transcription through a specific interaction between the ERE motif and a putative novel transcriptional factor.

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