Identification of an Epidermal Growth Factor Receptor Transcriptional Repressor*

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Identification of the factors controlling transcription of the epidermal growth factor (EGF) receptor gene is essential for understanding regulation of the EGF receptor and its overexpression in human carcinomas. In this study, we have identified a 60-base pair (bp) region (-918 to -858) relative to the AUG translation initiation codon in the EGF receptor 5' promoter that functions as a cis-acting EGF receptor transcriptional repressor (ETR). This fragment also acted as a repressor when linked to the thymidine kinase promoter. Gel mobility shift assays demonstrated that trans-acting factors bind to 60- and 18-bp fragments. Competition and chloramphenicol acetyltransferase assays with oligonucleotides containing mutations and deletions in this region indicate that the TTCCAGGG sequence (-877 to -870) is required for binding as well as repressor activity. While the ETR-protected region contains consensus sequences for the E2F binding site, no competition was observed with an E2F binding fragment. However, DNA-protein blot analysis indicates that both the 60- and 18-bp fragments specifically bind a 128-kDa polypeptide in extracts from HeLa or A431 human epidermoid carcinoma cells. These results suggest that a novel transcription factor(s) negatively regulates EGF receptor gene expression through binding to the ETR element.

The epidermal growth factor receptor (EGFR)† has extensive homology with the erbB oncogene product of the avian erythroblastosis virus (1, 2). Overproduction of the EGFR has been detected in several types of cancers (3-12), and overexpression of the EGFR can lead to epidermal growth factor-dependent transformation (13, 14). These results indicate that the EGFR gene function can act as an oncogene. In general, the production of EGFR mRNA correlates directly with EGFR levels on the cell surface (9). Although overexpression of EGF receptors in a number of tumors results from gene amplification (3-11), overexpression of EGF receptors in a variety of other tumors such as ovarian, cervical, and kidney tumors results from transcriptional or posttranscriptional mechanisms (9). Thus, transcriptional control undoubtedly plays a major role in the regulation of the EGFR gene. However, the mechanism by which this occurs is not well understood.

The promoter of the EGFR receptor gene lacks a "TATA box" and "CAAT box" but contains multiple "GC boxes" and multiple transcription initiation sites (15). An EGF receptor-specific transcription factor binds to an element located at position -248 to -233 bp relative to the AUG translation initiation codon (16). Partially purified EGF receptor-specific transcription factor stimulates in vitro transcription of the EGF receptor promoter 5-10-fold and appears to act only on promoters that lack TATA elements (17, 18). Cooperative distal upstream and downstream elements have been identified (19) as well as additional sequence motifs of potential importance (20, 21). Another factor that recognizes G+C-rich DNA sequences in the EGF receptor promoter and represses transcription has been characterized (22). This 91-kDa protein, termed GCF, binds strongly to two upstream regions of the EGF receptor promoter between -270 and -225 bp and weakly to a region between -150 and -90 bp. A 36-bp proximal element (-112 to -77) mediates stimulatory responses to EGF, phorbol 12-myristate 13-acetate, and cyclic AMP and acts in an orientation-independent manner (23). However, unlike transcriptional elements in most promoters, most of these elements are located downstream of the major in vivo transcription initiation site (15).

Although many transcription factors have been isolated and shown to stimulate transcription (24-31), relatively few transcriptional repressors have been described in eukaryotes. Furthermore, very little is known concerning how transcriptional repressors are involved in the suppression of proto-oncogenes. To address this question, we analyzed the regulation of the EGF receptor gene. In this study, we identified and characterized a novel repressor sequence located between -884 and -866 bp upstream of the translation initiator codon ATG in the EGF receptor promoter. Using Southwestern (DNA-protein blotting) analysis, we also identified a potential trans-acting repressor, a 128-kDa polypeptide that binds to this element in HeLa and A431 cells.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Chloramphenicol Acetyltransferase (CAT) Assays**

- Deletion mutants pERCAT4,5 were constructed as described (20). To test the effect of the ETR element on the thymidine kinase promoter, the 60-bp (StuI-BglII) DNA fragment was first treated with Klenow polymerase to generate blunt ends. The fragment was then ligated to a BamHI linker, digested, and subcloned into the BamHI site of ptkCAT (52), which contains the CAT gene driven by the herpes simplex virus thymidine kinase promoter. Two clones were isolated: pERkCAT1, which had the StuI-BglII DNA fragment in a 5'- to 3'-orientation, and pERkCAT2, which had the StuI-BglII DNA fragment in a 3'- to 5'.

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orientation. The chimeric plasmids of oligomer and tkCAT were constructed as follows. The oligonucleotides with XbaI site at 5’ end and BamHI site at 3’ end were synthesized and subcloned into the XbaI-BamHI sites of ptkCAT (32). Constructs containing the EGF receptor gene (ptkCAT) (32). Constructs containing the AP1, AP2, AP3, SP1, and NFVCTF binding sites were from Stratagene.

RESULTS
Identification of the ETR, a Cis-acting Repressor in the EGF Receptor Promoter—To identify regions required for EGF receptor gene expression, a series of deletion fragments of the human EGF receptor gene promoter ligated 5’ to the bacterial chloramphenicol acetyltransferase gene was tested for promoter activity by transfection into CV-1 African green monkey kidney cells, NRK fibroblasts, human HeLa cells, and human A431 epidermoid carcinoma cells. Functional analysis by CAT assay revealed the presence of both positive and negative regulatory elements. Among a series of CAT-EGF receptor promoter deletion constructs, pERCAT4 (Fig. 1) expressed relatively low CAT activity when transfected into CV-1, NRK, HeLa, or A431 cells. Transfection of a construct containing a further deletion, pERCAT5, resulted in an approximately 100% increase in CAT activity (Fig. 1). These results suggest that the region between StuI and BglII may contain a negative cis-acting element and that negative trans-acting factors may interact with the 60-bp StuI-BglII fragment to repress EGF receptor gene transcription.

To test whether the 60-bp StuI-BglII fragment will act as a repressor for a heterologous promoter, we constructed chimeric plasmids that contain the StuI-BglII fragment immediately 5’ to a thymidine kinase promoter that drives a reporter CAT gene (ptkCAT) (32). Constructs containing the StuI-BglII fragment in both orientations were isolated (pERtkCAT1 had a 5’- to 3’-orientation, and pERtkCAT2 had a 3’- to 5’-orientation (Fig. 1B)). When the constructs and ptkCAT were transfected individually into HeLa cells, repression of CAT activity by the 60-bp StuI-BglII fragment was observed for both pERtkCAT1 (80% decrease) and pERtkCAT2 (60% decrease) compared with the control, ptkCAT (Fig. 1B). We have termed this EGF receptor transcriptional repressor the ETR.

Trans-acting Factors Interact with the Cis-acting Element—To examine whether trans-acting factors specifically bind to the...
ETR sequence, we performed gel mobility shift analyses using the end-labeled 60-bp fragment (Fig. 2, lane 1) bound to proteins from CV-1, A431, or HeLa cell nuclear extracts (Fig. 2, lanes 2, 10, and 11). In these assays, a single DNA binding activity was observed that was specific for the 60-bp sequence. Complexes of identical mobility were also observed with nuclear extracts prepared from NRK cells.4 When nuclear extracts were incubated in the presence of a 100-fold molar excess of unlabeled 60-bp fragment, formation of the labeled complex was efficiently inhibited (cf. CV-1 in Fig. 2, lane 3). In contrast, neither a 100-fold molar excess of pBR322 DNA fragments, the SP1 binding site, the AP1 binding site, nor a 64-bp DNA fragment that contains AP1, AP2, AP3, and NF1/CTF binding sites competed for the binding activity (Fig. 2, lanes 8 and 9). These data indicate that the 60-bp fragment forms a specific DNA-protein complex.

Factor-binding Sequence of the ETR Element—The binding site(s) of the trans-acting factor(s) on the 60-bp fragment was localized by DNase I footprinting analysis and gel retardation assays. A 3′ end-labeled DNA fragment, which contains the 60-bp fragment and BamHI linker sequences at two ends, was used in DNase I footprinting assays (see Fig. 3A). In three independent assays, two protected regions were consistently detected over a 20-bp region (from −889 to −870). The first protected sequence (ATTTC) and the second protected sequence ATTTCCAGGG were separated by 5 bp.

Additional evidence for the importance of this region in binding of the trans-acting factor(s) was provided by gel retardation assays using probes of sequential deletions (Fig. 3B, lanes 1–6). A431 nuclear extracts were incubated with three DNA fragments that were 3′ end-labeled on the coding strand and digested by BamHI and TaqI, yielding 60-, 38-, and 16-bp probes. Gel retardation analyses revealed that the 60- and 38-bp probes, but not the 16-bp probe, formed DNA-protein complexes (Fig. 3B, lanes 2, 4 versus 6). The complex formed with the 38-bp fragment was smaller than the 60-bp complex, suggesting that the 60-bp fragment may have more than one protein binding site. The TaqI cleavage that generated the 16-bp fragment was in the middle of the second DNase I-protected sequence (ATTTC/GAGGG), consistent with protein binding at this site. Taken together, these results suggest that sequences contained within a 10-bp region (from −879 to −870) are crucial to the formation of a retarded protein-DNA complex.

To more precisely localize the repressor element, synthetic oligomers containing mutations or deletions within the 38-bp region were linked to the ptkCAT reporter gene and analyzed for repressor activity. The results are summarized in Fig. 4. The most repressor activity (60%) was observed with a 25-bp fragment (−884 to −860) and a 19-bp region within this fragment (−884 to −866). Further mutation of this 19-bp fragment resulted in partial or total loss of repressor activity. Thus, random sequence replacements of contiguous 8-bp segments resulted in 35% (ptkM2) and 25% (ptkM3) repression. Random sequence replacement of an 8-bp segments (ptkM4, −877 to −870) resulted in complete loss of repression. Mutation of 3 bp (ptkM1) within this 8-bp segment only slightly lowered repressor activity, illustrating the importance of the other 5 residues. These results indicate that the 19-bp sequence AGATGATTTCAGGGTGCT is sufficient, and the 8-bp sequence TTTCCAGGG is required for ETR repressor activity.

To confirm these results, competitive gel retardation studies were done using the 60- and 19-bp fragments as well as synthetic oligomers containing mutations within the 19-bp fragment. A specific loss in binding of the nuclear binding protein(s) to the 32P-labeled 60-bp fragment was obtained with a 100-fold molar excess of either the unlabeled 60-bp or unlabelled 19-bp fragments (Fig. 5A). A similar result was obtained when the 19-bp fragment was used as the 32P-labeled probe (Fig. 5B). In both cases, when the mutated 19-mer oligonucleotides (M1–4) were used as competitors, no significant loss of binding beyond...
A 38 mer s-5'GGCGCTATTCCGATGATTGCCAGGGTCGGTACAAA-3'
28 mer CCCTCATTCCGATGATTGCCAGGGTCGGTACAAA
25 mer AGATGATTGCCAGGGTCGGTACAAA
14 mer AGATGATTGCCAGGGTCGGTACAAA
M1 AGATGATTCCAGTGGTCGAAA
M2 AGATGATTCCAGTGGTCGAAA
M3 AGATGATTCCAGTGGTCGAAA
M4 AGATGATTCCAGTGGTCGAAA

| Relative CAT Activity | Repression(%) |
|----------------------|--------------|
| pIkCAT               | 75±2         | 25           |
| pM3tkCAT             | 75±2         | 25           |
| pM4tkCAT             | 100±1        | 0            |

Fig. 4. Repression of CAT activity by oligonucleotides. A, sequence comparison of the oligonucleotides inserted into the pCAT vector. The three stars above the sequence in M1 indicate the position of the three point mutants. The underlined sequences in M2, M3, and M4 indicate replacements in the 19-bp oligonucleotides by the random sequence GCAGTCTC. B, schematic maps of the CAT constructs used in this experiment. Arrow indicates the orientation of oligomers relative to the thymidine kinase promoter. The three stars inside the arrow of pM1tkCAT indicate the three point mutations. The black boxes inside the arrows of pM2tkCAT, pM3tkCAT, and pM4tkCAT represent replacements in the 19-mer by the random sequence GCAGTCTC. Relative CAT activity and repression (%) are shown at the right side.

that obtained with a nonspecific pBR322 fragment was observed. The E2F binding site oligonucleotide of the c-myc promoter didn’t compete significantly for binding either.

Identification of Polypeptides That Interact with the 60- and 19-bp Sequences—To identify proteins that bind to the negative element within the EGF promoter, Southwestern (DNA-protein blotting) analysis was performed (Fig 6). Nuclear extracts fractionated by SDS polyacrylamide gel electrophoresis were transferred to nitrocellulose and probed with the 32P-labeled 60-bp DNA fragment or a control probe (a 34-bp BamHI-HindIII DNA fragment of pUC19). A 128-kDa protein in extracts from both HeLa cells and A431 cells bound selectively to the 60-bp probe (Fig. 6, left). In some preparations of A431 cell extracts, the 60-bp probe reacted also with an additional 28-kDa polypeptide that may represent a degraded fragment (data not shown). No binding to the control probe was observed (Fig. 6, middle). Similarly, when the 32P-labeled 19-bp DNA fragment was used as a probe, only a 128-kDa protein was detected in A431 nuclear extracts (Fig. 6, right). These results indicate that the 60- and 19-bp fragments that contain the ETR element bind to at least one potential trans-acting factor.

DISCUSSION

In this study, we identified and characterized a novel repressor region in the human EGF receptor promoter. Deletion analysis, competitive gel mobility shift assays, and thymidine kinase promoter expression assays identified a 19-bp fragment (~884 to ~866) as the region containing the repressor binding site. Mutational analysis of this region demonstrated that the sequence TGGAGGG is required for ETR activity. Mutations of 3 bp within this sequence reduced, but did not eliminate, the ability of this oligomer to act as a repressor. Similar decreases in binding efficacy have been observed with oligomers mutated at 3 residues within enhancer or repressor regions of genes such as neu, an EGF receptor-related gene (39). Mutations of this region in the 19-bp fragment effectively eliminated competition or formation of the protein complex with the ETR element. Both the 60- and 19-bp fragments bound specifically to a 128-kDa protein in at least two cell types, suggesting that this protein may function as the ETR.

The level of repression observed in these studies (2–4-fold) is consistent with the levels reported for other proto-oncogenes or growth factor receptors. For example, deletion analysis of the fos promoter region yielded at most a 3-fold change and in general less than a 2-fold effect (40). The GCF repressor that acts on the EGF receptor (22) gave a 2-fold repression upon cotransfection. TGF-β repression of the trans/intramobility gene was 2-fold (41). It is difficult to assess the actual potency of the ETR as a repressor of EGF receptor transcription, because basal levels of repression observed in an untreated cell line probably do not reflect the importance of this region under in vivo conditions, where repressor or enhancer proteins may be present at very different levels. It is becoming clear that gene regulation is an integration of a number of effects resulting from interactions of factors from multiple sites on the promoter. It is noteworthy in this regard that the repressor element that we have identified is proximal to at least two other potential regulatory elements. Thus, to understand the regulation of the EGF receptor gene, it is important to dissect and characterize all of these elements.

The 19-bp fragment containing the ETR element also has a potential E2F binding site. The sequence TTTGAGGG (~878 to ~871) corresponds closely to the E2F binding site (TTTCGGGC) (42). Whether the 128-kDa protein corresponds to previously identified proteins remains to be determined. The 128-kDa protein is higher in molecular weight than most factors previously described including YY1 and E2F, which are approximately 68- and 54-kDa, respectively (43, 42). If E2F is a repressor that interacts with the ETR element through binding to the retinoblastoma gene product (44, 45), then E1A should bind retino-blastoma gene product and relieve the repression. However, cotransfection of an E1A expression vector with the pERCAT constructs into NRK cells did not alter the CAT activity of the EGF receptor promoter in the 60-bp region (data not shown). In addition, an oligomer, which contains the E2F binding site of the c-myc promoter did not block formation of either the ETR-19-bp DNA fragment complexes. These results suggest that the ETR is a novel transcriptional repressor distinct from E2F.

The potential complexity of the 60-bp fragment suggests that there may be more than one site of regulation within this domain of the EGF receptor. In addition to a potential E2F-like site, this region contains a YY1-like site and a TGF-β inhibitory element. CCTCATTG (~893 to ~886), a sequence that is just upstream of the 19-bp sequence but is present within the 38-bp fragment, is similar to the YY1 element (CNNCATTG). This element was originally identified as the binding site of the repressor protein YY1 in the adenovirus-associated P6 promoter (43). A site that is present in the 60-bp sequence but partially missing in the 38-bp fragment is the sequence QAGTCGCC, a TGF-β inhibitory-like element similar to that described previously for the trans/intramobility gene (41).5

By using a series of deletion CAT constructs, we have shown that this 60-bp region on the EGF receptor promoter is transcriptionally activated in response to TGF-β1 in NRK cells.5 Treatment of cells with TGF-β1 results in loss of specific protein binding to this 60-bp fragment, consistent with a model in which TGF-β1 prevents binding of a repressor(s) to the EGF receptor promoter. There are at least two possible sites in this region that may mediate TGF-β action, the ETR element and the TGF-β inhibitory element-like element. Whether TGF-β1 treatment activates EGF receptor transcription by altering binding of proteins to the TGF-β inhibitory element, the ETR

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1-100 kDa

73 kDa

43 kDa

128 kDa

Fig. 5. A, competition for ETR binding to the 60-bp fragment. The 3'-labeled 60-bp probe DNA was incubated with no protein (lane 1) or 2 µg of enriched A431 cell nuclear extract (lanes 2–9). Specific DNA-binding protein complexes (B) and free probe (F) are indicated. Competition of binding complexes was performed with a 100-fold molar excess of unlabeled probe (lane 3), pBR322 (lane 4), 19-bp oligomer (lane 5), and mutated oligomers M1–M4 (lane 6–9) as indicated. B, competition for ETR binding to the 19-bp oligomer. The 5'-labeled 19-bp probe DNA was incubated with no protein (lane 1) or 2 µg of enriched A431 cell nuclear extract (lanes 2–10). Specific DNA-binding proteins (B) and free probe (F) are indicated. Competition of binding complexes was performed with a 100-fold molar excess of 60-bp fragment (lane 3), pBR322 (lane 4), unlabeled 19-bp oligomer (lane 5), mutated oligomers M1–M4 (lane 6–9), or the E2F binding site oligomer of the c-myc gene promoter (lane 10) as indicated.

Fig. 6. Southwestern blot analysis of proteins that bind to the ETR element. Enriched nuclear extracts were fractionated on an 8% SDS-polyacrylamide gel, transferred by electroblotting onto a nitrocellulose membrane, and probed with the indicated 32P-labeled probes. Left, A431 and HeLa nuclear extracts probed with the 32P-labeled 60-bp fragment. Middle, HeLa nuclear extract probed with either the 32P-labeled 60-bp fragment or a control probe (a 34-bp BamHI-HindIII DNA fragment of pUC19). Right, A431 nuclear extract probed with either the 32P-labeled 19-bp oligomer or the mutated M4 oligomer. The relative positions (kDa) of the prestained molecular weight markers are indicated at the left. The molecular mass of the binding protein is indicated on the right.

repressor element, or both elements is presently under investigation.

The results reported here demonstrate that the ETR acts as a repressor for the thymidine kinase promoter, which has a TATA box, as well as for the TATA-less EGF receptor promoter in a number of cell types. Functional analysis revealed that the ETR-mediated repressor activity is present not only in human but also in rat NRK and African green monkey CV-1 cells. In addition, the cis-acting repressor element in both the 19- and 60-bp sequences was able to act with the heterologous thymidine kinase promoter, further substantiating the physiological relevance of this region. Therefore, this region and the protein that binds it are likely to be important not only for EGF receptor regulation but for other genes as well.

While the human EGF receptor gene is known to be overexpressed in certain tumors and cancer cell lines (3–12), only A431 cells had amplified EGF receptor genes. In a recent study of kidney tumors, we have found over-expressed EGF receptor transcripts without gene amplification in clear cell sarcoma of the kidney, congenital mesoblastic nephroma, and anaplastic Wilms’ tumor.3 The studies reported here raise the possibility that an ETR or its binding site could be a target for mutations that cause human EGF receptor gene overexpression in cancer cells.

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