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Identification and characterization of a regulated promoter element in the epidermal growth factor receptor gene

(transcription/DNA-binding protein/oncogene)

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ABSTRACT **We have identified a 36-base-pair proximal element (−112 to −77 relative to the AUG translation initiation codon) in the epidermal growth factor receptor 5′ region that functions as a promoter; mediates inductive responses to epidermal growth factor, phorbol 12-myristate 13-acetate, and cyclic AMP; and acts in an orientation-independent manner. This region functions as an enhancer when transferred to a heterologous promoter containing a TATA box. Mutations within the 36-base-pair region alter function as assayed by reporter gene expression in recipient cells. A protein has been identified that demonstrates appropriate binding specificity to mutant DNA sequences that correlates with promoter activity observed *in vivo*. On the basis of DNA binding characteristics and size, the identified protein appears distinct from several previously identified transcription factors known to bind to G+C-rich regions.**

The epidermal growth factor (EGF) receptor is an important mediator of cell growth and development (for reviews, see refs. 1–3). Accordingly, the cell-surface concentration of EGF receptors is highly regulated by transcriptional as well as posttranscriptional mechanisms and overexpression of either ligand or receptor is correlated with cell transformation and oncogenesis (4–8). The transcriptional mechanisms governing EGF receptor expression are poorly understood because this gene belongs to a class of promoters that lack typical TATA or CAAT elements (9, 10). TATA-less or "housekeeping" promoters are generally characterized by high G+C content and multiple transcriptional start sites (11–13).

A partial description of EGF receptor promoter elements indicates complexity in the regulation of EGF receptor gene expression. EGF receptor-specific transcription factor (ETF) binds to an element located at position −248 to −233 base pairs (bp) relative to the AUG translation initiation codon (14). Partially purified ETF stimulates *in vitro* transcription of the EGF receptor promoter 5- to 10-fold and appears to act only on promoters that lack TATA elements (15, 16). Cooperative distal upstream and downstream enhancer elements have been identified (17) as well as additional sequence motifs of potential importance (18, 19). Another factor that recognizes G+C-rich DNA sequences in the EGF receptor promoter and represses transcription has been characterized (20). This 91-kDa protein termed GCF binds strongly to two upstream regions of the extended EGF receptor promoter between −270 and −225 bp and weakly between −150 and −90 bp.

We have described a proximal 134-bp region (−153 to −19 bp) that serves as a promoter and mediates inductive responses to several environmental signals including EGF, phorbol 12-myristate 13-acetate (PMA), and cAMP (21). Deletional and mutational analyses identify a 36-bp promoter element (−112 to −77 bp) within the proximal 134-bp region. This element retains inductive responses to EGF, PMA, and cAMP and displays characteristics of an enhancer. DNA affinity chromatography and cross-linking data identify a protein that binds to this region with specificity that correlates with *in vivo* activity.

METHODS

Construction of Reporter Vectors and Measurement of Reporter Gene Activity. The EGF receptor 5′ region constructions were prepared by ligation of inserts into the luciferase expression vector pSVOALΔ5′ (22) or a luciferase expression vector containing the herpes simplex thymidine kinase (TK) promoter region (−81 to +52 bp) (23). The identity of each construction was established by dideoxynucleotide sequencing (24) and multiple isolates of each construction were examined for activity.

Plasmid DNA was transfected as a calcium phosphate precipitate (25) and luciferase activity was measured as described by de Wet *et al.* (22). Luciferase activities were normalized with respect to β-galactosidase activity and expressed as light units of luciferase activity per A_{420} unit of β-galactosidase activity (26). Vector background was <10 light units of luciferase activity per unit of β-galactosidase activity.

DNA Affinity Chromatography and UV Cross-Linking. HeLa nuclear extract was prepared from suspension cultures (27) and applied to a phenyl-Sepharose column in buffer A (20 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$/0.5 mM EDTA/1 mM dithiothreitol) with 1.5 M KCl. The column was washed with buffer A containing 1 M KCl and eluted with 50 mM KCl in the same buffer.

Oligonucleotides representing sequences −112 to −84 of the EGF receptor gene (EGFP) and −412 to −385 of the transforming growth factor α (TGF-α) 5′ region (TGFP) (28) were coupled to CnBr-activated Sepharose 4B (Pharmacia) (29). HeLa nuclear extract was applied to the TGFP oligonucleotide affinity column in buffer B (25 mM Tris·HCl, pH 8/10 mM $MgCl_2$/5 mM EDTA/1 mM dithiothreitol) in 0.1 M KCl. The column was washed with 0.2 M KCl in buffer B and eluted with 0.75 M KCl in the same buffer. Unadsorbed protein was applied to the EGFP oligonucleotide affinity column, which was washed with 0.1 M KCl in buffer B and eluted with 0.75 M KCl in the same buffer. The eluates were dialyzed and concentrated in Centricon 10 units (Amicon).

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Abbreviations: EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; ETF, EGF receptor transcription factor; GCF, G+C-rich DNA binding factor that represses transcription; RPF1, EGF receptor promoter factor 1; TK, thymidine kinase; TGF-α, transforming growth factor α.

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For UV cross-linking assays, a probe representing the −112 to −77 region of the EGF receptor promoter was prepared by annealing synthetic oligonucleotides and using the Klenow fragment of DNA polymerase I to fill in with [α-^{32}P]dATP, 5-bromo-2′-deoxyuridine triphosphate, dCTP, and dGTP. The oligonucleotides were designed such that BrdUTP was incorporated at either position −91 on the plus strand or −101 on the minus strand. The AP2 binding site probe represented residues −188 to −167 of the human metallothionein IIA gene 5′ region. Binding reactions for DNA cross-linking assays were performed in reaction volumes of 20 μl containing 10 mM Tris·HCl (pH 7.4), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 4% (vol/vol) glycerol, 100–500 ng of poly(dI-dC), and 10,000–35,000 cpm of labeled DNA probe (≈100 pM) without or with the indicated concentrations of competitor DNA. Column fractionated HeLa nuclear extract was added to the reaction mixture and the samples were incubated for 25 min at 23°C. Each reaction was irradiated with 300-nm UV light for 10 min. Samples were digested with 1 unit of *Staphylococcus aureus* nuclease for 30 min at 37°C, boiled in Laemmli sample buffer (30) for 5 min, and electrophoresed on SDS/10% polyacrylamide gels.

RESULTS

Identification of a Proximal Promoter Element of the EGF Receptor Gene. We have previously described inducible activity of the EGF receptor 5′ promoter region and localized full inductive responses to a 134-bp (*Not* I/*Sst* I) fragment (21). This portion of the EGF receptor promoter retains 20% of basal activity relative to the 1100-bp *Hin*dIII/*Sst* I fragment as measured by reporter gene expression but deletion of the 134-bp *Not* I/*Sst* I region reduced activity of the remaining fragment by >50%. The 134-bp fragment was examined further to determine regions responsible for basal activity (Fig. 1*A*). Deletion of 5′ sequences (−153 to −105 bp) and 3′ sequences (−49 to −19 bp) reduced activity to 65% and 38% of the parental control, respectively. The −55 to −19 bp element, which contains a single *in vivo* proximal start site, exhibited only minimal activity (4%) when placed in the luciferase expression vector. These results indicate that the 134-bp-inducible fragment of the human EGF receptor promoter contains at least two functional domains, a minimally active downstream element (−55 to −19 bp) that acts to augment an upstream element (−104 to −46 bp) of greater activity.

To determine functional boundaries of the active −104 to −46 bp element, oligonucleotides designed to successively delete sequences from the 3′ end of this region were subcloned into the luciferase expression vector. Because the *Ava* I restriction site was close to two *in vivo* transcriptional start sites (9, 10), the oligonucleotides were designed to extend 5′ relative to the *Ava* I site. Fig. 1*B* shows that addition of 8 bp 5′ of the *Ava* I site restored activity to equal the −153 to −50 bp fragment. Deletion of the 3′ sequences −76 to −46 bp failed to diminish activity. Unlike the 134-bp *Not* I/*Sst* I fragment, the 36-bp element spanning −112 to −77 bp was fully active in either orientation and, in addition, displayed enhanced activity when placed in multiple copies in the reporter vector; two and three copies of this region increased activity 10-fold and 30-fold, respectively. RNase protection analysis of RNA isolated from cells transfected with constructions containing the −112 to −77 bp region identified transcription start sites within this fragment similar to those described *in vivo* at residues −110 and −106 (9, 10) (data not shown). These results demonstrate that the 134-bp inducible fragment of the EGF receptor promoter contains a proximal element (−112 to −77 bp) that retains basal promoter activity and displays several properties of an enhancer.

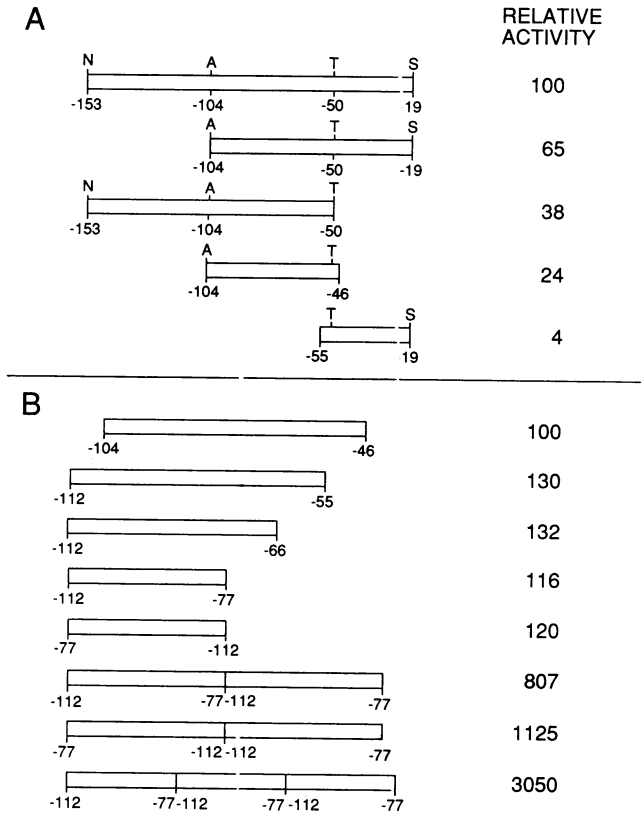

FIG. 1. Deletional analysis of the proximal promoter region of the EGF receptor gene. HeLa cells (10^7) were transfected with 10 μg of the indicated EGF receptor 5′ region luciferase construction and 10 μg of cytomegalovirus promoter-β-galactosidase expression vector and maintained in Dulbecco's modified Eagle's medium/F-12 medium containing 5% calf serum. Values shown represent the mean of luciferase activities obtained from triplicate cultures normalized with respect to β-galactosidase activity and are expressed as a percentage of the −153 to −19 bp fragment (49,130 ± 1740 light units per β-galactosidase unit) (*A*), or the −104 to −46 bp fragment (7990 ± 1423 light units per β-galactosidase unit) (*B*). Similar relative activities were determined in at least three separate experiments and multiple isolates of each construction were tested for activity. N, *Not* I; A, *Ava* I; T, *Tth*111 I; S, *Sst* I.

Induced Expression via the EGF Receptor Proximal Promoter Element. The inducible activities of the core promoter and the minimally active downstream element were determined and compared to the inductive responses of the parental 134-bp EGF receptor 5′ region (Fig. 2). The 134-bp region was responsive to EGF, PMA, and dibutyryl-cAMP with 2-fold, 7-fold, and 4-fold increases in luciferase activity, respectively. The 3′ element (−55 to −19 bp), which had only 4% of the basal activity of the larger fragment, retained a 3.5-fold response to PMA, but it was no longer responsive to the other agents. The −112 to −77 bp region was inducible by all three compounds and, although basal activity was elevated by multiple copies of this fragment, similar increases in relative inductive responses were observed. Because the −112 to −77 bp element has sequence similarity to the recognition site for the transcription factor AP2, this region was transfected into HepG2 cells, which do not express AP2 (31), and inductive responses were measured. The basal activity of this construction was the same in HepG2 and HeLa cells (24,174 ± 562 and 26,583 ± 3291 light units per unit of β-galactosidase, respectively), indicating that AP2 was not required for maintaining basal expression of this region. AP2 mediates inductive responses to both PMA and cAMP (32). In AP2-deficient HepG2 cells, the 36-bp element retained responsiveness to PMA, but not to cAMP. Observed

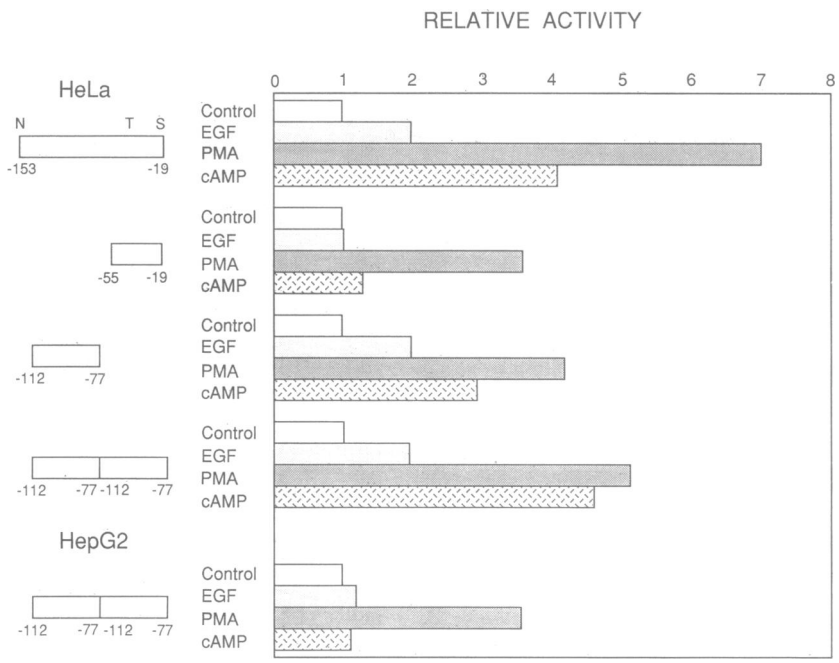

FIG. 2. Inductive responses of functional regions within the EGF receptor gene proximal promoter. HeLa and HepG2 cells were transfected with the indicated constructions and cells from each transfection were pooled and subcultured to eliminate variability due to initial transfection efficiency. Quadruplicate cultures were treated for 24 hr with the indicated compounds in bovine serum albumin (1 mg/ml) in Dulbecco's modified Eagle's medium/F-12 medium. Control, no hormone treatment; EGF, 10 nM; PMA, 10 nM; Bt_2cAMP, 1 mM. Induced responses are expressed relative to the untreated control for each construction and represent relative activities observed in three to five separate experiments.

PMA responses were thus not mediated through AP2, but a role for AP2 in cAMP responses is not excluded. No response to EGF was observed because HepG2 cells lack EGF receptors (21). These results indicate that the −112 to −77 bp promoter element of the EGF receptor gene retains inductive responses and its basal activity does not require AP2.

Mutational Analyses of Promoter and Enhancer Functions of the EGF Receptor Proximal Element. Further deletions and internal mutations of the −112 to −77 bp promoter element were made to examine regions required for basal activity (Fig. 3*A*). Removal of 3′ sequences −83 to −77 did not affect activity, indicating that promoter activity is located in the 29-bp element −112 to −84 (Fig. 3*B*). Deletion of −93 to −84 bp sequences greatly impaired activity as did deletion of −112 to −99 bp sequences (Fig. 3*B*). Internal mutations designed to reduce the G+C content and disrupt potential protein binding sites in this region were also examined. The 5′ mutation (M1) resulted in a reproducible increase in basal activity compared to the parental fragment. In contrast, the middle mutation (M2) severely reduced activity to 26% of control. The 3′ mutation (M3) was intermediate in its effects, reducing promoter activity to 45% of control. A sequence from the G+C-rich promoter

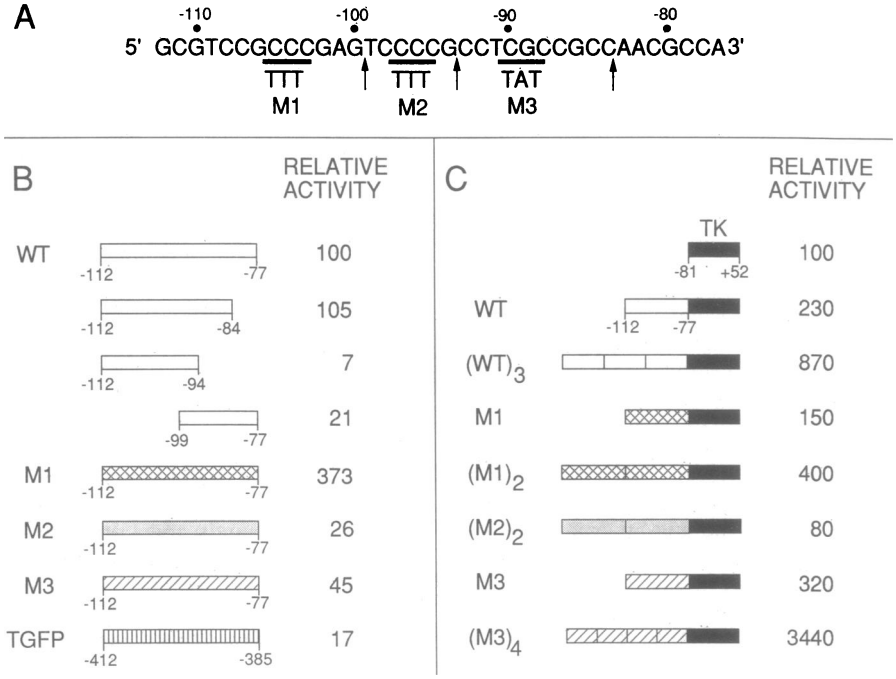

FIG. 3. Mutational analysis of the 36-bp proximal promoter element of the EGF receptor gene. (*A*) Sequence of the 36-bp proximal promoter element is shown and mutations designated M1, M2, and M3 are indicated by heavy lines. Arrows below the sequence mark the sites of 5′ and 3′ deletions. Dots and numbers above the sequence indicate base pairs relative to the AUG codon. (*B*) HeLa cells were transfected with the indicated EGF receptor promoter element–luciferase constructions. Values shown represent the mean of normalized luciferase activities, and relative activity is expressed as a percentage of the −112 to −77 bp element (6468 ± 143 light units per β-galactosidase unit). TGF-α 5′ sequences were compared to the wild-type (WT) EGF receptor −112 to −77 bp region. Multiple isolates of each construction were measured in three independent experiments. (*C*) HeLa cells were transfected with the indicated EGF receptor element–TK promoter–luciferase constructions. Values shown represent the mean of normalized luciferase activities and relative activity is expressed as a percentage of the −81 to +52 bp TK promoter.

of the TGF-α gene, which is identical in 21 of 27 residues to the EGF receptor core promoter sequence, exhibited minimal promoter activity (Fig. 3*B*), confirming specificity of the EGF receptor 5′ sequence.

In addition, we examined the ability of the −112 to −77 bp proximal promoter element and mutations of this region to function within the context of a heterologous promoter (Fig. 3*C*). In contrast to the ETF, this region enhanced activity of the TK promoter (−81 to +52 bp) 2- to 3-fold (single copy) or 8- to 9-fold (three copies). The activities of internal mutations assayed as enhancers in the chimeric constructions were compared to their independent promoter activities. The EGF receptor elements containing the mutations M1 and M3 functioned to enhance TK promoter activity with M3 > M1, while the middle mutation M2 was inactive even when present in two copies.

Identification of a Protein That Interacts with the Proximal Promoter Element of the EGF Receptor Gene. UV cross-linking assays identified only one component of nuclear extract, termed receptor promoter factor 1 (RPF1), that demonstrated high-affinity, sequence-specific binding to the EGF receptor promoter element. Fig. 4*A* (lane 1) shows that two proteins of ≈90 and ≈50 kDa are bound to the EGF receptor 5′ element. Addition of unlabeled competitor DNA decreased binding of the 50- but not the 90-kDa protein (lane 2). The binding specificity was further defined by competition with EGF receptor 5′ element DNAs that contain the mutations shown in Fig. 3*A*. Both M1 (lane 3) and M3 (lane 5) competitor DNAs specifically decreased binding of the 50-kDa protein, whereas M2 (lane 4) and TGF-α 5′ (lane 6) DNAs were only weakly active as competitors at ≈25-fold molar excess. The 90-kDa protein was poorly and nonspecifically competed by wild-type and mutant DNAs.

Specificity was analyzed in more detail by using increasing concentrations of competitor DNAs (Fig. 4*C*). Competitor wild-type DNA at low concentrations effectively decreased cross-linking of the 50-kDa protein to labeled DNA. M1 and M3 were also effective competitors in the order wild type > M3 > M1. M2 DNA failed to significantly decrease RPF1 binding, even at the highest concentration tested. The 90-kDa protein was blocked by competition with higher concentrations of all the DNAs tested, confirming lack of specificity of binding.

Direct binding of 50-kDa RPF1 to radiolabeled mutant DNA probes in gel mobility-shift assays also showed impaired binding of RPF1 to M2 but not to M1 or M3 DNA, confirming the UV cross-linking competition results (data not shown). These findings suggest that residues −102 to −91 are involved in the binding of RPF1 to the EGF receptor promoter; additional nucleotides in the −112 to −84 sequence may also be involved.

Although AP2 did not appear involved in basal promoter activity, a role for AP2 in induction of the EGF receptor proximal element by cAMP was suggested by *in vivo* transfection results. Because AP2 is also a 50-kDa protein, we tested whether RPF1 was distinct from AP2 by using competition with a high-affinity AP2 binding site, the distal basal level enhancer, from the human metallothionein IIA gene 5′ region (32). The distal basal level enhancer was inactive as a competitor for 50-kDa RPF1 when tested at concentrations up to 10-fold molar excess; this concentration of unlabeled wild-type probe gave complete competition (Fig. 4*B*).

RPF1 was fractionated from HeLa nuclear extract by sequence-specific DNA affinity chromatography. The inactive TGF-α 5′ element was used as a first column to adsorb nonspecific G+C binding proteins. Protein unadsorbed to the TGFP column was applied to a second column composed of the −112 to −84 EGF promoter sequence coupled to Sepharose. The eluted and nonadsorbed fractions were UV cross-linked to the 36-bp EGF receptor proximal element. The labeled proteins are shown in Fig. 5*A* without nuclease digestion to give a more accurate indication of their relative quantities. After nuclease digestion (Fig. 5*B*), they migrate closer to their apparent molecular mass.

The RPF1 selectively bound to the EGFP column (Fig. 5, lanes 4 and 4′). Much of the 90-kDa protein in the extract was retained by the first, nonspecific TGFP column, while the RPF1 was not adsorbed. The second column of EGFP resin effectively sequestered RPF1 from the extract (lanes 3 and 3′). Residual 90-kDa protein in the extract was bound less well by the EGFP column and was also present in the flow-through.

DISCUSSION

The best defined eukaryotic RNA polymerase II promoters consist of proximal TATA elements that direct the transcription start site and distal sequences which bind proteins that regulate transcriptional activity (33). Two types of promoters lacking TATA elements have been identified. In one type, an

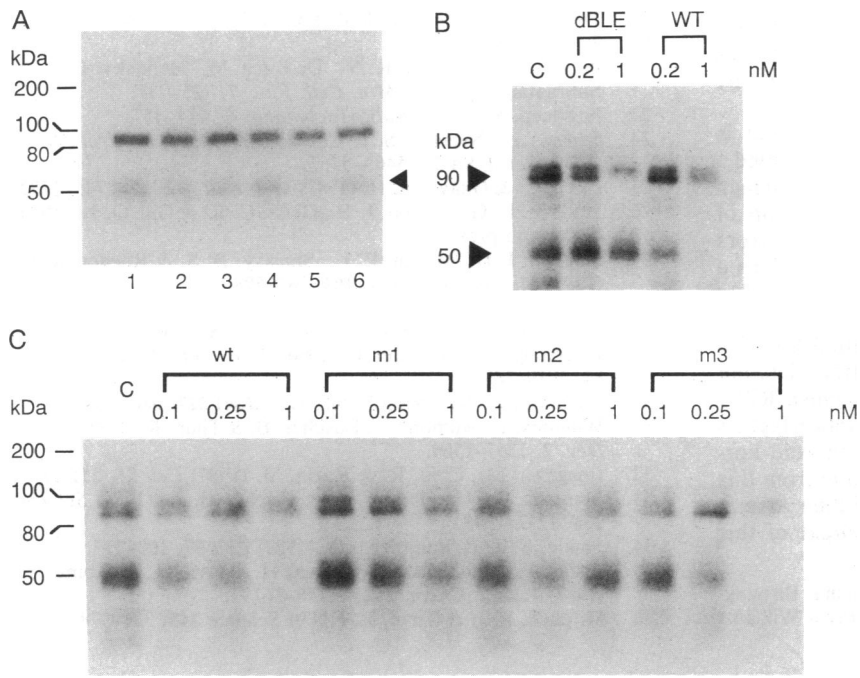

FIG. 4. UV cross-linking of protein to the EGF receptor promoter element. Phenyl-Sepharose fractionated HeLa nuclear extract was incubated with EGF receptor promoter element DNA labeled with [α-^{32}P]dATP and BrdUTP along with the indicated competitor DNA. (*A*) Samples contained the plus-strand BrdUTP-labeled probe and either no competitor (lane 1) or 2.5 nM EGFP (lane 2), M1 (lane 3), M2 (lane 4), M3 (lane 5), or TGFP (lane 6). The 50-kDa RPF1 is indicated by the arrowhead. (*B*) Samples contained the minus-strand BrdUTP-labeled probe and either no competitor (lane C), 0.2 or 1 nM high-affinity AP2 binding site probe [lanes dBLE (distal basal level enhancer)], or 0.2 or 1 nM EGFP (lanes WT). (*C*) Samples contained the minus-strand BrdUTP-labeled probe and either no competitor (lane C) or 0.1, 0.25, or 1 nM EGFP (wt) or mutant probes (M1, M2, or M3).

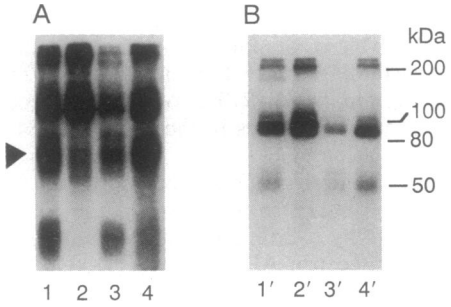

FIG. 5. DNA sequence-specific affinity chromatography. HeLa nuclear extract was applied sequentially to TGFP and EGFP columns. Nonadsorbed and eluted fractions were UV cross-linked to EGF receptor proximal element probes and electrophoresed on Laemmli gels without (*A*) or with (*B*) *S. aureus* nuclease digestion. Probes were UV cross-linked to 3 μg of crude nuclear extract protein (lanes 1 and 1′) or 1 μg of protein in the TGFP column eluate (lanes 2 and 2′), in the EGFP column flow-through (lanes 3 and 3′), or in the EGFP column eluate (lanes 4 and 4′). In *A*, RPF1 is indicated by the arrowhead.

element, the "initiator," dictates accurate basal transcription from a start site within its sequence (34). The second type of TATA-less promoter is the G+C-rich class found in a number of genes. In contrast to TATA-containing promoters, which accurately initiate at a single site, only a few of these G+C-rich promoters initiate transcription from a single site (28, 35) while many demonstrate multiple transcription start sites (11–13).

We have identified a 36-bp element in the TATA-less G+C-rich EGF receptor 5′ sequence that functions as a transcription initiation site when placed in front of a marker gene. This element also displays many characteristics of an enhancer; it is active in either orientation, multiple copies increase transcription synergistically, and it increases the activity of the TATA-containing TK promoter when placed directly upstream. With a minimal TK promoter (−37 to +52 bp), the 36-bp element gives robust 80-fold and 160-fold enhancement in reporter gene activity when added as one and two copies, respectively (J.X., unpublished data). The EGF receptor promoter element mediates inductive responses generated by EGF receptor, protein kinase C, and cAMP-dependent protein kinase pathways. AP2, which can mediate induction by cAMP and PMA, appears not to be involved in this element's response, as gene expression from this promoter was increased by PMA in HepG2 cells, which lack AP2. Another transcription factor that can be activated by multiple signal pathways appears to be involved.

The activity of the EGF receptor promoter element is probably mediated by multiple factors. We have identified a nuclear protein of ≈50 kDa in HeLa cells that binds with high affinity and specificity to the element. The disruption of RPF1 binding by a series of internal mutant probes mirrors the effect of mutations on the activity of the element as a promoter and an enhancer. Although the M2 mutation would disrupt a weak Sp1 binding site, identified by DNase footprinting (9), gel mobility-shift assays with purified Sp1 (36) fail to show any effect on binding by this mutation (data not shown). Differences in molecular weight distinguish RPF1 from previously identified EGF receptor transcription factors ETF and GCF (14–16, 20). Retinoic acid and thyroid hormone receptors also bind and inhibit transcription from this element (26). Multiple regulatory elements and their assembly will have to be considered within the context of this organization of a G+C promoter.

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