Molecular Cloning and Characterization of a Transcription Regulator with Homology to GC-binding Factor*  

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The epidermal growth factor receptor (EGFR) plays an important role in cell growth and development (5–7). Overexpression of the EGFR can lead to epidermal growth factor-dependent transformation (8, 9). Overproduction of EGFR has been detected in several types of cancers due to gene amplification (10). Overexpression of EGFR transcripts in a variety of other tumors such as ovarian, cervical, and kidney tumors results from transcriptional or posttranscriptional mechanisms (11). A variety of agents have been shown to increase EGFR gene expression (12–14). Repression of EGFR gene transcription by different agents has also been reported (15, 16). Transcriptional control must play a major role in regulation of EGFR gene expression.

The promoter of the EGFR gene lacks a TATA box and CAAT box but contains multiple GC boxes and multiple transcription initiation sites. A number of regions in the promoter have been identified that bind nuclear factors (17–19). Furthermore, Sp1, wild type p53, EGFR transcription factor, and AP2 have been shown to activate EGFR gene transcription (20–23). Three repressor proteins, EGFR transcriptional repressor, GC-binding factor (GCF), and the Wilms’ tumor suppressor, also bind to sites within the EGFR promoter (24–26).

A cDNA for GCF was isolated by screening an A431 expression library with GC-rich sequences from the EGFR promoter. GCF is a 91-kDa protein that binds to three upstream sites of the EGFR promoter. Two are between bp 270 and 225, and the other site is between 150 and 90 relative to the translational start site. Cotransfection experiments have shown that GCF can repress transcription of the EGFR promoter and several other growth-related gene promoters such as transforming growth factor-α and insulin-like growth factor II (27).

The cDNA for GCF hybridizes to three mRNA species of 4.5, 3.0, and 1.2 kb (28). The GCF cDNA contains 2.8 kilobase pairs and is likely to encode the 3.0-kb mRNA. The larger 4.5-kb mRNA may result from homology to another gene or possibly from alternative splicing of the GCF gene. Since the 5’ portion of the GCF cDNA that encodes the DNA binding region hybridizes very strongly to the 4.5-kb mRNA, it is possible that the cDNA for this mRNA also encodes a DNA binding protein. In this report, we present results on isolation of a cDNA that hybridizes to the larger mRNA and has homology to the 5’-end of the GCF cDNA. We also show that the protein encoded by this mRNA binds to the EGFR promoter and represses the activity of the EGFR, SV40, and RSV promoters.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U69609.

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MATERIALS AND METHODS

Cell Culture—Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and antibiotics. Medium was removed, and cells were washed with phosphate-buffered saline without Ca2+ and Mg2+ prior to RNA isolation. KB cells were treated with 100 μm nortabol 12-myristate 13-acetate (Sigma) in Me2SO (Aldrich) for up to 7 h in the above medium. 

Isolation and Sequencing of GCF2 cDNA Clones—The 282-bp GCF cDNA fragment (1–282) was labeled with [α-32P]dCTP and used as a hybridization probe to screen an ovarian carcinoma (OVCAR-3) cell cdna library constructed in Uni-Zap XR (Stratagene). Positive clones were purified and phagemid bodies were excised using R408 helper phage (Stratagene). The clones were sequenced with an Applied Biosystems model 373A automated DNA sequencer. Sequence comparisons were performed with BLAST and PROSITE using the default parameter to search the National Cancer for Biotechnology Information nonredundant protein and DNA data bases (34, 35).

5′-Rapid Amplification of cDNA Ends (RACE)—5′-RACE Ready cDNA cDNAs were purchased from CLONTECH (Palo Alto, CA). GCF2-specific primers were selected using Oligo 4.0 (National Biosciences). Nested primers were used to enhance specificity. The 5′-RACE product, detected after primary and secondary amplifications, was purified by agarose gel electrophoresis, subcloned into pCRII (Invitrogen), and sequenced. The RACE products contained homology to the GCF2 cDNA clones and extended to the 5′-end. The full-length GCF2 cDNA was composed of restriction fragments.

In Vitro Translation—The open reading frame of GCF2 was amplified by PCR and subcloned into pCITE2A (Invitrogen). Protein was synthesized in vitro in the presence of [35S]methionine with the coupled transcription/translation system (TNT) from Promega (Madison, WI). Translated products were analyzed on SDS-polyacrylamide gels (36).

Bacterial Expression and Purification—The GCF2 open reading frame was cloned into pQE60 (Qiagen) at the BamHI site after the addition of BamHI linkers to the open reading frame by PCR. The new plasmid, pcGCF2-His, was sequenced to check for mutations and used to transform JM109. JM109 cells containing pcGCF2-His were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 0.7 for 4.5 h. Cells were harvested and resuspended in sonication buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl). Cells were subjected to two cycles of freezing and thawing followed by treatment with lysozyme (1 mg/ml) for 30 min on ice. The sample was then sonicated (1-min bursts/1-min cooling/200–300 watts) on ice and treated with 10 μg/ml RNase A for 15 min. After centrifugation at 10,000 × g for 20 min, the supernatant was mixed with nickel-nitrilotriacetic acid resin for 60 min at 4 °C. The mixture was loaded into a column and washed with sonication buffer followed by sonication buffer plus 0.5 mM imidazole and sonication plus 40 mM imidazole. The GCF2-His protein was eluted in sonication buffer plus 0.5 mM imidazole and examined by SDS-polyacrylamide gel electrophoresis. Fractions containing GCF2-His were dialyzed versus a buffer containing 20 mM HEPES, pH 7.9, 20 mM KCl, 1 mM MgCl2, 2 mM dithiothreitol, and 17% glycerol. Dialyzed samples were stored in aliquots at −80 °C.

dNuc Mobility Shift Assays—Mobility shift assays were performed as described previously (17). A double-stranded oligonucleotide containing the putative GCF2 binding site was prepared by annealing two complementary oligonucleotides containing nucleotides −249 to −229, 5′-CGGGCAGCCCCGGCCAGCG-3′ and 5′-CGTGGCCGGCGGGCTCAG-3′, in a buffer containing 10 mM Tris, pH 8.0, 500 mM NaCl, and 1 mM EDTA. Equimolar amounts of the complementary oligonucleotides were incubated with a 15-mM EGFR promoter (1–675) in a heat block at 95°C. The heat block was allowed to cool to room temperature, and the sample was desalted on a G-25 Sephadex column. The double-stranded oligonucleotide and EGFR promoter fragments were end-labeled with 32P using T4 polynucleotide kinase and γ-ATP. For the gel shift analysis, end-labeled EGFR promoter fragments or double-stranded oligonucleotide were incubated with GC2F2-His or nucleo extract at room temperature (23 °C) for 15 min in the presence of 10 mM Tris, pH 7.5, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 50 μg/ml poly(dI-dC)-poly(dI-dC) and 4% glycerol. Nuclear extract from OVCAR-3 cells was prepared as described earlier (37). When competition assays were performed, unlabeled EGFR fragments or mutated GCF2 binding site oligonucleotides were incubated with purified buffer for 5 min prior to the addition of the labeled oligonucleotide. The mutated GCF2 binding site oligonucleotides were purchased as single-stranded DNA strands from Genosys Biotechnologies and annealed as described above. The AP2 binding site oligonucleotide was purchased from Promega. Samples (10 μl) were loaded onto a 5% polyacrylamide gel and subjected to electrophoresis at 150 V for 2 h. The gel was stained with 0.1% TBE (89 mM Tris, 88 mM NaCl, 2 mM EDTA, pH 8.3) as running buffer. After electrophoresis, gels were transferred to Whatman 3 MM paper and exposed to Kodak XAR film with intensifying screens at −70 °C.

dNucase I Footprinting—dNucase I footprinting was performed according to Dynan et al. (38). The EGFR promoter fragment (−771 to −16) was labeled at the Hind III site, and a 553-base pair (−569 to −18) fragment was isolated after restriction digestion with TagI. GC2F2-His was prepared as described earlier. AP2 and Sp1 were obtained from Promega.

Transfections and Chloramphenicol Acetyltransferase (CAT) Assays—African green monkey kidney cells (CV-1) or OVCAR-3 were seeded at 5 × 104 cells/100-mm dish incubated overnight at 37 °C in a 5% CO2 incubator. For each transfection, 2–10 μg pCMVGCF2 and 2 μg of promoter-CAT DNA were mixed in 1.5 ml of Opti-MEM (Life Technologies), and a precipitate was formed using lipofectamine (Life Technologies) according to the manufacturer’s recommendations. The cells were washed with serum-free Dulbecco’s Eagle’s medium, and complexes were applied to the cells for 5 h. Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum was added, and cells were incubated overnight. Medium was changed the following day and cells were grown for an additional 24 h. Cells were harvested and extract was prepared as described previously (39). CAT activity was assayed in extracts using the CAT assay kit from Promega. Transfection efficiency was monitored by measuring β-galactosidase activity from an RSV-β-galactosidase reporter plasmid construct that was also co-transfected. The EGFR promoter-CAT constructs, pERCAT9, pERCAT10, and pERCAT19, were prepared previously by our laboratory (17). The SV40 early promoter CAT construct, pSV2CAT, and Rous sarcoma virus long terminal repeat CAT construct, RSVCAT, were obtained from Dr. Bruce Howard (National Institutes of Health) (39). The cytomegalovirus immediate early gene 3 promoter CAT construct, EMVCAT, was obtained from Dr. Sumita Deb (University of Texas Health Science Center at San Antonio) (40). The products containing mutations in the GCF2 binding site were prepared by first using PCR with mutated oligonucleotide primers and a HindIII restriction site to generate the desired mutation in the promoter. The fragment was digested with HindIII and ligated into the HindIII site of pSVOCAT. The orientation of the promoter was determined by restriction enzyme mapping, and fidelity of the sequence was confirmed by DNA sequencing using the Applied Biosystems model 373A automated sequencer.

Mass Spectroscopy—GC2F2-His was diluted 1:10 into 50% acetonitrile, 0.1% trifluoroacetic acid in water. BSA (Sigma) in the same solvent was mixed with the dilute GC2F2 by trial and error to yield a comparable mass spectrum signal. A ratio of five parts GC2F2 to one part BSA worked well. The protein/BSA sample was then mixed with an equal amount of sinapinic acid solution (Hewlett Packard). The sample was analyzed on the Hewlett Packard model 2052A matrix-assisted laser desorption ionization, time of flight mass spectrometer. The GC2F2 mass was averaged from eight trials, with each trial combining the data of 20–30 laser shots.

RESULTS

Differential Hybridization of GCF cDNA Fragments—We have previously reported that GCF cDNA hybridizes to three mRNA species of 4.5, 3.0, and 1.2 kb in numerous cell lines. We were also interested in examining the GCF hybridized differently to the three mRNA species (21). A fragment containing nucleotides 1–282 and a fragment containing nucleotides 314–961 were prepared by PCR, labeled with 32P, and used in Northern blot hybridization analysis. Fragments were prepared by PCR to avoid the stretch of 21 adenosines in the GCF cDNA that would hybridize to many RNAs. The fragment containing nucleotides 1–282 hybridized to an mRNA of ap-
proximately 4.5 kb with virtually no hybridization to other mRNAs (Fig. 1A). In contrast, a fragment containing nucleotides 314–961 hybridized very strongly to mRNAs of 3.0 and 1.2 kb but only slightly to the 4.5-kb mRNA. This was true using RNA from both A431 and KB epidermoid carcinoma cells (Fig. 1B). Identical results were obtained using RNA from ovarian carcinoma cell line (OVCAR-3) and a T-cell lymphoma cell line (HUT-102) (data not shown).

**GCF2 cDNA Isolation**—To isolate the cDNA corresponding to the larger mRNA, a cDNA library prepared from ovarian carcinoma cell mRNA (OVCAR-3) was screened using the fragment containing nucleotides 1–282 as a probe. Fourteen positive cDNA clones were isolated and sequenced. The two largest clones, O (1.4 kilobase pairs) and Q (2.6 kilobase pairs) were determined to contain all of the DNA sequences present in the 14 clones (Fig. 2). The O clone sequence was found to contain an open reading frame that extended to the 5'-end of the clone. To obtain additional sequence present at the 5'-end of the cDNA, RACE was performed. The end of the open reading frame was obtained with an additional 126-bp 5'-untranslated region. The sequence of the combined cloned cDNAs consists of 3523 bp with an open reading frame of 2256 nucleotides (Fig. 3). The GCF2 cDNA has a region of sequence homology with the GCF cDNA of 309 bp (98% identity) (Fig. 4). The remainder of the sequence has no further significant homology to GCF or any other sequence found in GenBank. The deduced protein sequence of GCF2 is shown in Fig. 3. The amino acid sequence indicates the presence of potential phosphorylation sites for cAMP-dependent kinase, calcium-dependent kinase, and tyrosine kinase. Also, the presence of an N-linked glycosylation site and a nuclear localization sequence is predicted.

**GCF2 mRNA Characterization**—To determine the size of mRNA that the GCF2 cDNA hybridizes, Northern blot hybridization analysis was performed. Poly(A)+ RNA isolated from D551 (normal human fibroblast), A431 (epidermoid carcinoma), KB (epidermoid carcinoma), OVCAR-3 (adenocarcinoma), T98G (glioblastoma), Raji (Burkitts’ lymphoma), and HUT-102 (T-cell lymphoma) cells was transferred to nitrocellulose and probed with a radiolabeled GCF2 cDNA probe. As compared with an RNA size ladder, a 4.2-kb mRNA hybridized to the GCF2 cDNA (Fig. 5). If comparison is made to ribosomal RNA migration, the size would be 4.5 kb, which was the original size estimate. The 4.2-kb GCF2 mRNA was detected in all cell lines with higher levels found in Raji, T98G, and HUT-102 cells.

**Production and Analysis of GCF2 in Reticulocyte Lysates and Escherichia coli**—As described above, the open reading frame of the GCF2 consists of 2256 residues and should encode a protein of 83 kDa. The open reading frame was cloned into the pCITE2A vector, and coupled *in vitro* transcription/translation was performed in the presence of radiolabeled methionine. The radiolabeled translation product was analyzed on an SDS-polyacrylamide gel. GCF2, made *in vitro* in reticulocyte lysates, migrates as a protein of 160 kDa, approximately twice the expected size (Fig. 6). The GCF2 open reading frame was also subcloned into a bacterial expression vector containing a His tag sequence. The protein was expressed in bacteria, and the His tag protein was purified on nickel-nitrilotriacetic acid resin. The purified GCF2-His tag protein was analyzed by SDS-polyacrylamide gel electrophoresis and was found to migrate the same as the protein made in reticulocyte lysates (Fig. 7). GCF2 has a pI of 4.4 and contains 22% acidic residues.

To further analyze the molecular mass of GCF2, mass spectrometry was performed. Matrix-assisted laser desorption ionization, time of flight mass spectrum analysis of GCF2-His gave an average mass of 83,960 Da. The percentage error of the internal standard BSA was 0.01%, and the error for the GCF2-His was estimated to be 0.05%, since its mass is outside the limits of the two-point linear calibration used with BSA (doubly charged mass = 33215.5 and singly charged mass = 66431).
DNA Binding Studies—The homology of the GCF2 cDNA and GCF cDNA is confined to the DNA binding region of GCF. To test whether GCF2 could bind to specific sites in DNA, DNase I footprinting experiments were performed. GCF2 was shown to bind to one site in the EGFR promoter located between -249 and -233 (Fig. 8). As controls, GCF bound to the same site. Amino acid sequence analysis of GCF2 revealed potential phosphorylation sites, N-glycosylation sites, and a putative nuclear localization signal. A homology alignment of GCF2 and GCF cDNAs using default parameters for the BestFit sequence analysis software package of the Genetics Computer Group demonstrated the sequence identity. Numbers to the left and right of the sequences represent the respective nucleotides of the cDNAs.
AP2 and Sp1 were used to footprint the promoter, and both have footprints that overlap the GCF2 footprint. To see if there were GCF2 binding sites of lower affinity and to confirm the DNase I footprinting results, gel electrophoretic mobility shift assays were used. Three EGFR promoter fragments were end-labeled and incubated with GCF2-His. Two fragments, 2384 to 2167 and 2105 to 216, bound GCF2 and exhibited altered mobility during polyacrylamide gel electrophoresis (Fig. 9). The binding affinity of GCF2 to the 2384 to 2167 fragment was 50–100-fold greater than to the 2105 to 216 fragment as measured by densitometric scanning of the retarded bands. Also, this difference in affinity was seen in competition experiments using the 2105 to 216 fragment (Fig. 10A) and the 2384 to 2167 fragment (Fig. 10B). An EGFR promoter fragment containing residues 167 to −105 did not bind GCF2 (data not shown). Oddly, there was no footprint detected between 2105 and 216. These results indicate that GCF2 binds with different affinities to the different sites. To further define the GCF2 binding site, site-directed mutations were placed at different locations throughout the binding site. Double-stranded oligonucleotides containing these mutations were used to compete with a radiolabeled GCF2 binding site oligonucleotide. The effect of these mutations is shown in Fig. 11. The mutated oligonucleotides that do not compete represent essential nucleotides in the binding site (Fig. 11, lanes 3–8). Mutated oligonucleotides that still compete contain changes that do not affect GCF2 binding (Fig. 11, lanes 9 and 10). Thus, the core of the GCF2 binding site is AGCCCCCGGCG.
effect of GCF2 on EGFR gene expression. GCF2 cDNA (pCMVGCF2) or the empty vector control (pCMV) was cotransfected with receptor plasmids containing the CAT gene under control of the EGFR promoter (pERCAT6), the SV40 early promoter (pSV2CAT), the Rous sarcoma virus long terminal repeat promoter (RSVCAT), or the cytomegalovirus IE3 gene promoter (CMVCAT). These promoter constructs were selected because they are all strong promoters and because GCF repressed the EGFR promoter but not the other three (25). As shown in Fig. 12, cotransfection with the GCF2 expression plasmid resulted in significant repression of the expression of three promoters (EGFR, SV40, and RSV) but not the CMV promoter. The control expression plasmid, pCMVGCF2R, in which the GCF2 cDNA is inserted in reverse orientation, had no effect on expression from any of these plasmids (data not shown). The extent of repression by GCF2 was similar for all three reporter plasmids, 3–4-fold at a 5:1 GCF2/CAT ratio, and was also seen when OVCAR-3 cells were transfected.

The effect of GCF2 on the EGFR promoter was further examined using EGFR promoter deletion constructs. Promoter constructs containing the major GCF2 binding site, pERCAT-6, pERCAT-7, pERCAT-8, and pERCAT-9, were repressed approximately 4-fold when cotransfected with pCMVGCF2 (Table I), pERCAT-10, pERCAT-14, and pERCAT-15, which do not
contain the major GCF2 binding site, were only slightly repressed, −1.5-fold. These results indicate that GCF2 repression of the EGFR promoter requires a binding site located between −384 and −167. When this site is mutated in a way to prevent GCF2 binding, the repression of the EGFR promoter is also lost (Fig. 13). The M2 mutation does not allow for GCF2 binding, and the EGFR promoter reporter construct containing the M2 mutation is not repressed by GCF2 in cotransfection experiments. Conversely, the M9 mutation, which does not affect GCF2 binding does not prevent repression by GCF2. Thus, there is a direct correlation of GCF2 binding and repression.

The binding site for GCF2 either overlaps or is in close proximity to binding sites for AP2 and Sp1. To examine the binding of proteins to this region, nuclear extracts from OVCAR-3 cells and a labeled GCF2 binding site oligonucleotide were prepared. A gel mobility shift assay was performed, and cold oligonucleotides were used to compete for the binding (Fig. 14). The M2 and M3 oligonucleotides competed for some but not all of the binding (Fig. 14, lanes 3 and 4). An oligonucleotide containing an AP2 binding site also competed for some but not all of the binding at a 100-fold molar excess (Fig. 14, lane 7). These results indicate that GCF2 and AP2 can bind to this region.

We have previously shown that increased EGFR gene expression due to phorbol ester treatment was mediated via AP2 (23). Since GCF2 and AP2 can bind to overlapping sites in the promoter, we examined the effect of phorbol ester treatment on GCF2 expression and compared it to EGFR expression. Total RNA was isolated from treated cells, and the expression of GCF2 and EGFR was analyzed by Northern blotting. GCF2 mRNA levels decreased as low as 10-fold during the time course (Fig. 15). In contrast, the EGFR mRNA increased approximately 5-fold during the same time frame. Also, the decrease in GCF2 mRNA preceded the increase in EGFR mRNA. Thus, there is an inverse correlation of GCF2 and EGFR expression following phorbol 12-myristate 13-acetate treatment.

**DISCUSSION**

Transcriptional repression is an important factor in the regulation of many genes. Transcription of specific genes can be modulated downward in various ways. Most of the steps required for activation of transcription can be altered by transcriptional repressors. Repressors fall into two basic categories, passive and active. Passive repressors down-regulate the activity of one or more transcriptional activators by competing for binding sites or by binding the activator. Active repressors have an intrinsic repressing activity and inhibit transcription initiation directly.

**GCF2 Has Homology to GCF**—In this paper, we describe a new repressor that has a DNA binding activity and represses the activity of the EGFR gene. The GCF2 cDNA was isolated and cloned based on homology to GCF. The initial 309 base pairs of the GCF cDNA are homologous to residues 1382–1690 of GCF2. There is a 98% identity, 305 of 309 base pairs, between the GCF and GCF2 cDNAs in this region. The region of homology is restricted to the 5'-end of the GCF cDNA but is internal, nucleotides 1382–1690, to the GCF2 cDNA.

**GCF2 Migrates with an Altered Mobility during SDS-Polyacrylamide Gel Electrophoresis**—The GCF2 deduced protein...
sequence contains a DNA binding and nuclear localization motif similar to GCF (Fig. 3). GCF2 expressed from the open reading frame migrates as a 160-kDa protein on SDS-polyacrylamide gels. However, the calculated molecular mass is 83 kilodaltons. This could be due to the acidic nature of the protein (p = 4.4 and 22% acidic residues) or to an unusual ability to form very stable dimers. α-70 from E. coli also migrates at a significantly higher molecular mass (90 kDa) on SDS-polyacrylamide gels as compared with the calculated molecular mass of 70 kDa (41). Production of protein from deletion mutants in reticulocyte lysates revealed that the altered migration during SDS-polyacrylamide gel electrophoresis is associated with the protein sequence between residues 490 and 530 (data not shown). This region includes the putative DNA-binding region and the nuclear localization signal. It contains a sequence stretch of residues where 11 out of 14 are lysine. Charge interactions between this region and acidic regions may result in a protein conformation that has an aberrant migration on SDS-polyacrylamide gels.

GCF2 binds DNA and Represses Transcription—GCF2 binds to EGFR promoter fragments with different affinities. The promoter fragments, −384 to −167 and −105 to −16, were labeled to similar specific activities, but GCF2 binding was stronger to the −384 to −167 fragment (Fig. 8). DNase I footprinting detected a single footprint in the −384 to −167 region. We have not been able to localize the GCF2 binding site in the −105 to −16 fragment due to a much lower affinity. The binding site detected by DNase I footprinting resembles binding sites for GCF. This is not unexpected, since there is sequence homology to the DNA binding region of GCF. The GCF2 binding site overlaps binding sites for Sp1 and AP2 (Fig. 8). Also, p53 has been reported to bind the EGFR promoter between −265 and −239 (42). Thus, this location is very important in the regulation of EGFR promoter activity by transcriptional activators. Binding of GCF2 may provide a mechanism to turn down promoter activity after activation or to prevent unwanted activation. This could be mediated through direct binding to DNA or through protein-protein interactions with Sp1, AP2, and p53.

GCF2 represses activity from three different promoters in cotransfection assays. Two of these promoters, EGFR and SV40, have GC-rich regions. The EGFR promoter has been shown previously to be repressed by GCF, while the SV40 promoter was shown not to be repressed (25). It is interesting to note that although the overall effect of GCF2 on the promoters was about the same, at lower GCF2/reporter ratios the effect was more evident on the SV40 and RSV promoters. This may be due to the greater activity of these promoters, which are 5–10-fold stronger than the EGFR promoter in CV-1 cells. GCF2 may be acting as either an active repressor or a passive repressor. In either case, it appears to be a general repressor, since it is active on both cellular and viral promoters. To determine whether GCF2 is an active or passive repressor requires detailed studies of DNA-protein interactions and protein-protein interactions. Recently, NAB1 was isolated and shown to be a repressor of NGFI-A and Krox20-mediated transcription (43). NAB1 interacts directly with the activators to prevent transcriptional activation. Nuclearin was recently purified and shown to bind to the B-motif of the α-1 acid glycoprotein and to repress transcription of the α-1 acid glycoprotein promoter in transfection assays (44). Analysis of GCF2 will aid in increasing understanding of gene regulation and in extending our knowledge of the mechanisms of action of eukaryotic transcriptional repressors.

The Relationship of the GCF and GCF2 cDNAs—One unresolved issue is the high degree of homology between the 5′-end of the GCF cDNA and residues 1382–1690 of the GCF2 cDNA. Attempts to isolate GCF cDNA clones from cell lines other than A431 have resulted in isolation of cDNAs that do not contain the GCF 5′-region (data not shown). Furthermore, we have not been able to isolate an identical GCF cDNA from an A431 cDNA library but have isolated additional cDNA clones that contain internal deletions. These cDNAs all contain 5′-ends that begin around nucleotide 320 of the GCF cDNA (data not shown). This indicates that the GCF 5′-region is possibly a cloning artifact or is a mutation found in A431 cells that have a number of chromosomal abnormalities. Also, P1 clones containing GCF genomic DNA sequences do not hybridize with the 5′-end of the GCF cDNA (data not shown). However, the melting temperature of the GCF cDNA around residue 320 is greater than 85 °C. This results in problems in amplifying the cDNA by PCR and may interfere with additional experimentation, including cDNA synthesis using reverse transcriptase. The origin of the GCF cDNA requires a more detailed analysis of gene structure that includes genomic clones from normal tissues and A431 cells.

EGFR levels are decreased by a number of factors including retinoic acid and nerve growth factor. Recently, it has been shown that the decrease of EGFR in PC12 cells is controlled at the transcriptional level (45). Also, correlating with the decrease in EGFR transcription is an increase in GCF2. In agreement with these studies is the correlation of a decrease in GCF2 mRNA and an increase in EGFR mRNA by phorbol 12-myristate 13-acetate reported in this study (Fig. 15). This suggests that GCF2 may have a physiological role in regulating EGFR transcription. Additional studies on GCF2 and EGFR expression will aid in understanding the complex nature of EGFR gene regulation.

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