Expression and Chromosomal Localization of the Gene for the Human Transcriptional Repressor GCF*

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GCF is a human transcriptional regulator that represses transcription of certain genes and is encoded by a 3-kilobase (kb) mRNA (Kageyama, R., and Pastan, I. (1989) Cell 59, 815–825). The expression of GCF was examined in a variety of clonal cell lines. The 3.0-kb GCF mRNA was found to be expressed at the highest level in HUT 102 cells (derived from a T-cell lymphoma). Elevated levels of the GCF mRNA were also noted in KATO III and AGS (gastric carcinomas), FEM-X (melanoma), and U266B1 (myeloma) cell lines. A human fibroblast cell line (WI38) did not express GCF mRNA, and no cross-hybridization to a mouse cell line (NIH 3T3) or monkey cell line (CV-1) could be detected. The GCF cDNA also hybridizes to RNA species of 4.5 and 1.2 kb. The 4.5-kb RNA has the same general expression pattern as the GCF mRNA. Hybridization of cellular RNA with various probes derived from the 3-kb cDNA revealed that the 4.5-kb RNA species only hybridizes to GCF cDNA probes from the extreme 5′ end. By using single-stranded RNA probes, hybridization to the three RNA species was detected with the antisense probe for the 5′ end (nucleotides 1–561). The single-stranded antisense probe for the region encompassing nucleotides 561–1692 hybridized to the 3.0- and 1.2-kb RNA species. The sense probes for these regions did not hybridize to these RNAs. The GCF gene was localized to a single locus, the chromosome 2 p11.1–11.2 region, by in situ hybridization. Treatment of human KB epidermoid carcinoma cells with phorbol 12-myristate 13-acetate (PMA) lead to a rapid induction of GCF RNA after 1 h and a decline to lower than control levels after 6 h. Epidermal growth factor receptor mRNAs were not increased by PMA until 2 h after treatment and were at their highest level only after GCF mRNAs were decreased. The 4.5- and 1.2-kb RNAs were also induced by PMA with the same kinetics as the GCF mRNA. These results show that the GCF gene is widely expressed in human tissues and cell lines and that the 4.5- and 1.2-kb RNAs have similar expression patterns.

The role of specific proteins in regulating gene expression via DNA binding interactions is now widely studied. DNA-protein interactions and protein-protein interactions have been shown to play an essential part in determining the level of gene products (1–5). Studies on transcription have clearly shown that genes encoding eukaryotic proteins contain complex groups of regulatory elements that modulate transcription. These cis-regulatory elements bind specific proteins. A combination of several cis-elements and binding proteins can generate various effects on the transcription of a specific gene, i.e. induction, repression, or maintenance of the basal level. In addition, it has been recognized recently that different proteins in the cell can bind or compete for the same DNA recognition sequences (6–9). These proteins can either bind at different times or compete for binding at the same time. The net effect on gene expression may depend on the affinity of the proteins for the binding site and the amount of each specific protein present at a given time.

Several proteins that bind to GC-rich sequences have been identified. Sp1 is a well studied transcription factor that binds to GC-rich sequences and activates transcription (10, 11). ETF, a transcriptional factor found to bind to the epidermal growth factor receptor promoter, is another protein that stimulates transcription through binding to GC-rich sequences, including some Sp1 binding sites (12, 13). Whether Sp1 and ETF compete for binding in some cases is not known at the present time. Other protein factors have been identified which bind to enhancer regions that are GC-rich (14, 15). In addition to these activator proteins, one repressor protein that binds to GC-rich sequences, termed GCF, has been identified (16). GCF binds and represses transcription of the epidermal growth factor (EGF) receptor, β-actin, and calcium-dependent protease promoters (16). This repression has been shown in vivo via transfection experiments and in vitro with a reconstituted cell-free transcription system. The DNA recognition sequence for GCF is similar to the binding sites for Sp1 and ETF.

To understand more about the relationship between these factors, we have further characterized the expression of GCF. We now report that GCF mRNA is readily detected in many human tissues and human cell lines and but not fibroblasts. We also have found two other RNA species that hybridize to the GCF cDNA. We have determined the chromosomal localization of the GCF gene and examined the induction of GCF mRNA. Our results indicate that GCF is widely expressed in human tissues and cell lines and may play an important role in regulating gene expression.

MATERIALS AND METHODS

Cell Cultures—Cells were maintained in medium supplemented with 10% fetal bovine serum (GIBCO). Medium was removed and

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1 The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; SDS, sodium dodecyl sulfate; PMA, phorbol 12-myristate 13-acetate; kb, kilobase pair(s).
cells washed with phosphate-buffered saline three times prior to RNA or DNA isolation.

**RNA Isolation and Blotting**—Total RNA was isolated by the acid-guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (17). Tissues were pulverized on dry ice prior to the addition of guanidinium thiocyanate. Tissue samples were homogenized by passage through a 20-gauge needle and RNA isolated after acid-phenol extraction. Total RNA was isolated from cultured cells by direct addition of the guanidinium thiocyanate to the dish. Cells were disrupted in a Dounce homogenizer and RNA isolated after acidification or DNA isolation. Cells were washed with phosphate-buffered saline three times prior to RNA isolation. Chomczynski and Sacchi (17). Tissues were pulverized on dry ice homogenized by passage through a 20-gauge needle and RNA isolated as above. Poly(A)+ RNA was selected from the total RNA population by oligo(dT)-cellulose chromatography (18). RNA concentrations were determined by measuring absorbance at 260 nm. RNA was fractionated on a 1% formaldehyde-agarose gel and transferred to nitrocellulose (19). Prehybridizations, hybridization, and washings were carried out as described previously (20). Labeled cDNA probes were prepared by nick translation (21) or random primer extension (22).

**RNA Probes and Hybridizations**—The EcoRI-SstI (1–561) fragment was subcloned into pGEM4Z that was restricted with EcoRI and SstI. The SstI-HindIII (561–1692) fragment was also subcloned into SstI-HindIII-cut pGEM4Z. RNA probes, sense and antisense, labeled with [α-32P]deoxycytidine triphosphate (32P-CTP) were synthesized using either SP6 or T7 polymerase. Northern blots were prehybridized at 60 °C for 2 h in a solution containing 50% formamide, 5 × SSC, 5 × Denhardt’s solution, 50 mM sodium phosphate, pH 6.5, and 200 μg/ml sheared salmon sperm DNA. Hybridizations were carried out using 106 cpm/ml RNA probe at 60 °C in a solution containing 50% formamide, 5 × SSC, 1 × Denhardt’s solution, 20 mM sodium phosphate, pH 6.5, 10% dextran sulfate, and 100 μg/ml sheared salmon sperm DNA. Filters were washed at 70 °C with 2 × SSC + 0.2% SDS followed by 0.1 × SSC + 0.2% SDS also at 70 °C. Filters were air dried and exposed to film.

**Chromosomal Localization**—Chromosomes were obtained from methotrexate-synchronized normal peripheral leukocytes cultured for 72 h in RPMI 1640 medium containing phytohemagglutinin, 15% bovine serum, and antibiotics. One- or 2-day-old chromosome preparations were used for in situ hybridizations. One microgram of GCF cDNA was nick translated with all four 3H-deoxynucleotides to a specific activity of 1.8 x 106 cpm/μg DNA. Chromosome preparations were treated with RNase for 1 h at 37 °C. After several washings in 2 × SSC and dehydration in a series of alcohols, chromosomes were hybridized with the GCF probe labeled with [32P]dCTP were synthesized using either SP6 or T7 polymerase. Northern blots were prehybridized at 60 °C for 2 h in a solution containing 50% formamide, 5 × SSC, 5 × Denhardt’s solution, 50 mM sodium phosphate, pH 6.5, and 200 μg/ml sheared salmon sperm DNA. Hybridizations were carried out using 106 cpm/ml RNA probe at 60 °C in a solution containing 50% formamide, 5 × SSC, 1 × Denhardt’s solution, 20 mM sodium phosphate, pH 6.5, 10% dextran sulfate, and 100 μg/ml sheared salmon sperm DNA. Filters were washed at 70 °C with 2 × SSC + 0.2% SDS followed by 0.1 × SSC + 0.2% SDS also at 70 °C. Filters were air dried and exposed to film.

**RESULTS**

**GCF mRNA**—To determine the types of cells expressing GCF we analyzed the levels of GCF mRNA in clonal cell lines (Figs. 1 and 2; Table I). Fig. 1 shows a Northern blot made with total RNA and probed with a 1.9-kb EcoRI fragment (1–1931) of the GCF cDNA at 42 °C for 16 h. Blots were washed with 2 × SSC + 0.1% SDS and 0.2 × SSC + 0.1% SDS at 60 °C. × SSC = 0.15 μM sodium chloride plus 0.015 M sodium citrate. The washed filters were exposed to film for 96 h. Lane 1, A431; lane 2, KB; lane 3, KATO III; lane 4, AGS; lane 5, Hs 746T. Arrows indicate the size of RNAs hybridizing to the GCF probe.

**GCF mRNA**—To determine the types of cells expressing GCF we analyzed the levels of GCF mRNA in clonal cell lines (Figs. 1 and 2; Table I). Fig. 1 shows a Northern blot made with total RNA and probed with a 1.9-kb fragment of the GCF cDNA. Two bands hybridize with the probe. The smaller is 3 kb in size and corresponds to the size of the original clone. Another RNA species of 4.5 kb is also detected and is usually present in greater amounts than the 3-kb GCF mRNA. GCF is present in 2 human epithelial carcinoma cell lines (A431 and KB, lanes 1 and 2, respectively) and at a higher level in two of the three human gastric carcinoma cell lines (KATO III and AGS, lanes 3 and 4, respectively). The highest level of GCF mRNA was found in HUT 102 cells (Fig. 2, lane 6) as compared with the AGS level (Fig. 2, lane 1). Levels of GCF mRNA similar to AGS were detected in total RNA from FEM-X and U266B1 cells (Fig. 2, lanes 5 and 7, respectively). No GCF mRNA was detected in total RNA from human fibroblast cells (WI38) (Fig. 2, lane 2). Total RNA from CV-1 cells (monkey) and NIH 3T3 cells (mouse) (lanes 3 and 4, respectively), did not cross-hybridize under the conditions used in these experiments (see “Materials and Methods”). Total RNA from other human epithelial cell lines including HeLa, Caki-1, SK-Hep and PLC/PRF5, was found to contain GCF mRNA (Table I). The 4.5-kb RNA was expressed with the same general pattern as the GCF mRNA but at higher levels. A human γ-actin probe acted as an internal control for RNA loading (data not shown). The data in Table I are adjusted for variation in RNA amounts as detected by the actin probe.

The hybridization of the 4.5-kb RNA species to the GCF cDNA probes was unexpected. To ascertain why this RNA species was not detected in our previous reports (16) we hybridized a Northern blot containing poly(A)+ RNA from A431 cells with the 1.9-kb EcoRI fragment labeled by nick translation (Fig. 3A) in one case and random primer extension (Fig. 3B) in the other case. The nick-translated probe hybridized predominantly to the 3.0-kb GCF mRNA whereas the random primer-labeled probe hybridized to the 4.5- and 1.2-kb RNA species in addition to the 3.0-kb GCF mRNA. The specific activity of the random primer extension label probe is approximately 1 order of magnitude greater than the nick-

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**Fig. 1.** Northern blot hybridization analysis of gastric carcinoma cell line RNAs. RNAs were isolated from epithelial (A431 and KB) and gastric carcinoma (KATO III, AGS, and Hs 746T) cell lines and Northern blot hybridization analysis performed as described under "Materials and Methods." The blot was probed by hybridizing with a 32P random primer-labeled 1.9-kb EcoRI fragment (1–1931) of the GCF cDNA at 42 °C for 16 h. Blots were washed with 2 × SSC + 0.1% SDS and 0.2 × SSC + 0.1% SDS at 60 °C. × SSC = 0.15 μM sodium chloride plus 0.015 M sodium citrate. The washed filters were exposed to film for 96 h. Lane 1, A431; lane 2, KB; lane 3, KATO III; lane 4, AGS; lane 5, Hs 746T. Arrows indicate the size of RNAs hybridizing to the GCF probe.

**Fig. 2.** Northern blot hybridization analysis of cell line RNAs. RNAs were isolated from cell lines and Northern blot analysis performed as described under "Materials and Methods." The GCF probe, hybridization, and washings are the same as in Fig. 1. The filter was exposed to film for 48 h. Lane 1, AGS; lane 2, W138; lane 3, CV-1; lane 4, NIH 3T3; lane 5, FEM-X; lane 6, HUT 102; lane 7, U266B1. GCF mRNAs are indicated by the arrows to the left of the figure.

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* N. C. Popescu, manuscript in preparation.
Expression of GCF mRNA and the 4.5-kb RNA species

The level of GCF mRNA expression was determined by Northern blot hybridization analysis. RNA expression is shown for the 4.5- and 3.0-kb RNA species. For tissues it is expressed relative to liver, and for cell lines, relative to Hs 746T. RNA loading was monitored by hybridization with an actin probe. ++, 4-5-fold higher; +++, 8-10-fold higher; ++++, 16-20-fold higher.

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![Fig. 3. Differential hybridization of a nick-translated and random primer extension-labeled GCF cDNA probe.](image)

Transcribed probe. Hybridization of the nick-translated probe to Northern blots containing total RNA gave a very weak hybridization signal, and therefore we used the random primer extension label probe for the total RNA Northern blot.

To define further the spectrum of GCF mRNA expression we examined various human tissues for the presence of GCF mRNA (Table I). Both the 4.5-kb RNA and GCF mRNA were present in all 15 tissues we examined (Table I). The highest level of expression of the 4.5-kb mRNA was found in esophagus with moderate expression in ovary, testes, and kidney. Low but detectable levels were found in the remaining tissues. The 3.0-kb GCF mRNA was detectable at low levels in all tissues. This indicates that GCF mRNA is present in many tissue types, as is the 4.5-kb RNA species.

To determine the relationship of the different mRNA species, we selected poly(A)⁺ RNA from total RNA isolated from KB cells and performed Northern blot hybridization analysis using fragments from different regions of the 3-kb GCF cDNA which were labeled by random primer extension (Fig. 4 and 5). When poly(A)⁺ RNA was used, RNA species of 4.5, 3.0, and 1.2 kb could be detected with probes that contain the 5' region of GCF (Fig. 4 A and B). However, cDNA probes that do not contain the 5' region hybridize to the 3.0- and 1.2-kb species but not the 4.5-kb species (Fig. 4, C-E, and Fig. 5). This suggests that the 5' end may contain sequences homologous to another gene or is alternatively spliced.

To rule out the possibility that one or more of the RNAs are transcribed from opposite strands of DNA, we probed Northern blots with single-stranded RNA probes. The RNA probe corresponding to the antisense strand for nucleotides 1–561 hybridizes to the 4.5-, 3.0-, and 1.2-kb RNA species (Fig. 6A). The RNA probe corresponding to the sense strand for nucleotides 1–561 did not hybridize to any RNA species.

**Fig. 4. Differential hybridization of GCF fragments to RNA.** Poly(A)⁺ RNA was isolated from KB cell total RNA, analyzed by Northern blot analysis, and probed with various random primer extension-labeled GCF fragments. Hybridizations and washes were the same as in Fig. 1. Filters were exposed to film for 18–36 h. A, EcoRI 1.9-kb fragment (1–1931); B, SstI 0.56-kb fragment (1–561); C, SstI 2.2-kb fragment (561–2804); D, EcoRI 0.5-kb fragment (1931–2453); E, EcoRI 0.4-kb fragment (2453–2845).

**Fig. 5. Summary of differential hybridization results with GCF probes.** A schematic of the GCF cDNA (5' to 3' to left to right) and fragments used to hybridize to RNAs. The presence (+), strong presence (++), or absence (−) of hybridization to specific RNA species is shown beneath each fragment. Fragment A, EcoRI, 1.9 kb (1–1931); fragment B, EcoRI-SstI, 0.56 kb (1–561); fragment C, SstI, 2.2 kb (561–2804); fragment D, EcoRI, 0.5 kb (1931–2453); fragment E, EcoRI, 0.4 kb (2453–2845).
were hybridized and washed as described under "Materials and Methods." 10 pg of RNA was electrophoresed on a formaldehyde-agarose gel and transferred to nitrocellulose. Filters were hybridized and washed as described under "Materials and Methods." A, nitrocellulose probed with antisense RNA to the 5' region containing nucleotides 1-561. B, nitrocellulose probed with antisense RNA to the region containing nucleotides 561-1692.

A total of 149 grains from 98 spreads with label on nonoverlapping chromosomes were localized on a 400-band ideogram (Fig. 7A). A total of 149 grains from 98 spreads with label on nonoverlapping chromosomes were localized on a 400-band ideogram (Fig. 7A). We observed specific accumulation of grains only on chromosome 2; 41 grains representing 27% of the total number of grains and 87% of the grains on chromosome 2 were on the short arm at bands p11.1-11.2 (Fig. 7, B and C). Grains on other chromosomes were randomly distributed. Based on grain distribution, we assign the location of the GCF gene to 2 p11.1-11.2.

PMA Induction—The role of GCF as a repressor in gene regulation is only beginning to be defined. Questions remain as to when it acts or how is it activated. Toward this end we examined GCF mRNA induction by treatment of KB cells with PMA. This compound, which induces EGF receptor gene expression, also increased the level of GCF mRNA (Fig. 8A). The time course of induction was rapid, peaking 5-fold above control approximately 1–2 h after treatment. This is in contrast to the EGF receptor mRNA induction, which peaked 10–15-fold higher than control levels 6 h after PMA treatment (Fig. 8B).

**DISCUSSION**

The regulation of gene expression involves the interaction of many different factors with cis-regulatory elements in the DNA. Genes respond to these interactions by increasing, decreasing, or maintaining their current level of expression. Protein factors that increase eukaryotic gene expression have been widely studied. However, eukaryotic repressors have not been as well studied.

Our laboratory has reported previously on the cloning of a human DNA-binding factor that represses transcription (16). This factor, termed GCF, bind to sites in the EGF receptor promoter, the β-actin promoter, and the calcium-dependent protease promoter. The binding sites in these promoters consist of a stretch of G residues (GGGGGG) with C residues interspersed at two locations. GCF represses transcription of the EGF receptor promoter in vivo and in vitro. The binding of GCF to DNA occurs through the interaction of the highly basic amino-terminal amino acids with the binding site in the DNA (16).

To extend our knowledge of GCF, we have examined the
expression of GCF mRNA in human tissues and cell lines. We found that two major species of GCF mRNA were present in total RNA of all tissues examined with the highest level in esophagus (Table 1). Other tissues expressed low to moderate amounts of GCF mRNA (Table 1). GCF mRNA was also found in all human cell lines examined except WI38 fibroblasts.

The presence of GCF mRNA in these tissues and cell lines shows that it is ubiquitous and could exist to regulate the expression of many different genes. We did not observe any correlation between the level of GCF mRNA and the level of EGFR receptor RNA. GCF mRNA levels were not changed by EGFR or serum deprivation, two factors known to affect changes in EGFR receptor RNA (39). However, upon treatment of KB cells with PMA, the level of GCF mRNA increased approximately 5-fold and then decreased. The level of EGFR RNA did not increase until the level of GCF mRNA decreased (Fig. 8). This is suggestive of a role of GCF in regulating the EGFR gene. In A549 cells, derived from a lung adenocarcinoma, 12-O-tetradecanoylphorbol 13-acetate, has been reported to repress EGFR gene expression. This repression has been localized to the region of the EGFR promoter containing the region from nucleotide -384 to -151 (25). This region contains two GCF binding sites (16).

The major finding of this study was the detection of three RNA species that hybridize to the GCF cDNA. The 3.0-kb RNA corresponds to the GCF cDNA we have cloned (16). Using high specific activity random primer extension-labeled probes, we additionally observed hybridization to RNA species of 4.5 and 1.2 kb. These RNA species were not detected with a lower specific activity nick-translated probe. The 4.5-kb RNA species hybridizes very strongly to the 5' portion of the GCF cDNA (nucleotides 1-561). However, it does not hybridize to the remainder of the GCF cDNA. It is plausible that the 4.5-kb RNA corresponds to a cDNA possessing a DNA binding region similar to that of GCF. It is clear that families of proteins exist with similar function and structure. Proteins have been described with arginine-rich motifs similar to the one found in the DNA binding region of GCF (26, 27). The cDNA sequences for these genes are somewhat similar to the GCF cDNA sequence in the DNA binding region (26, 27). This DNA binding region also contains a stretch of 21 deoxyadenosines. It is unlikely that this stretch is hybridizing to other RNAs since the hybrids can form at 60 °C in 50% formamide. The hybrids also are resistant digestion by RNase A (data not shown). The ultimate relationship between the GCF cDNA and any of these genes.

The EGFR receptor gene promoter contains binding sites for several nuclear proteins (12, 13, 16, 40-42). These proteins include the activator proteins Sp1 and E72 as well as the repressor GCF. The ultimate expression of the EGFR gene depends on interactions of these factors with the promoter and perhaps each other. Repression of EGFR gene expression may occur through several mechanisms, e.g., direct binding of repressors, competition for binding by repressors and activators, quenching or squelching (for review see 43). GCF may be involved in repression by any of these mechanisms. GCF may also act to return EGFR gene expression to a steady-state level after activation by other agents. Further analysis will provide insight into the process by which GCF represses transcription of EGFR and other genes and what relationship exists between GCF mRNA and the 4.5-kb RNA species.

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