THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 2, Issue of January 9, pp. 1032–1037, 1998

Printed in U.S.A.

The Cloning and Expression of a New Guanylyl Cyclase Orphan Receptor*

(Received for publication, August 4, 1997, and in revised form, October 22, 1997)

Stephanie Schulz‡§, Barbara J. Wedel‡¶, Anne Matthews‡, and David L. Garbers‡‖

From the ‖Howard Hughes Medical Institute and the ‡Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9050

A novel membrane form of guanylyl cyclase (GC-G) has been identified through the isolation of a full-length cDNA clone; it is predicted to contain an extracellular ligand binding domain, a single transmembrane segment, and intracellular protein kinase-like and cyclase catalytic domains. That GC-G represents a guanylyl cyclase was confirmed by both transient expression in COS-7 cells and stable expression in H293 cells. Endogenous cyclic GMP concentrations of transfected or stable cells, however, were much higher than control cells, suggesting an inability of the cells to effectively regulate GC-G cyclase activity. Of six Cys residues found within the extracellular domain of guanylyl cyclase-A (GC-A), the receptor for atrial natriuretic peptide, five are conserved within GC-G. Ligands for the other cyclase receptors, nevertheless, failed to stimulate GC-G expressed in transient or stable cells, suggesting that the unknown ligands possess a structure different from the natriuretic peptides or heat-stable enterotoxins. ^{125}I-ANP also failed to bind to H293 cells overexpressing GC-G. Based on Northern hybridization, mRNA for GC-G was predominantly expressed in lung, intestine, and skeletal muscle. Using the candidate gene approach to potentially define function, the gene for GC-G was mapped to the distal region of mouse chromosome 19 (syntenic with human chromosome 10q), but no human genetic defect has been ascribed to the GC-G locus. The finding of a new membrane form of guanylyl cyclase in peripheral tissues suggests the existence of another family or subfamily of ligands that signal through elevations of cGMP.

Of the six membrane forms of guanylyl cyclase in the mammal, three are receptors for known ligands, whereas three remain presumed orphans (1). The first mammalian cyclase cDNA isolated encoded a receptor (guanylyl cyclase-A (GC-A)[1]) for atrial natriuretic peptide (ANP) (2, 3). Disruption of the GC-A gene yields mice with a salt-resistant form of elevated blood pressure (4), and it has been subsequently suggested that GC-A is the primary means by which the heart communicates with the kidney to cause natriuresis/diuresis when vascular volume is acutely increased by intravenous infusion (5). The guanylyl cyclase-B receptor (GC-B) appears to bind C-type natriuretic peptide (CNP) with highest affinity, and although its normal functions remain unclear (6, 7), it has been suggested to regulate cell growth (8, 9) and blood pressure (10, 11). GC-C represents the receptor for heat-stable enterotoxins (STa) and possibly two endogenous peptides, guanylin and uroguanylin (12–14). It has been suggested to normally regulate intestinal fluid secretion (15), but disruption of the GC-C gene has not resulted in an adverse phenotype when mice are raised in a disease-free environment. In fact, GC-C null mice are resistant to STa- or enterotoxigenic bacteria-induced diarrhea (16). The three orphan guanylyl cyclase receptors are expressed in sensory tissues, two in the retina (17–19) and one in olfactory neuroepithelium (20). GC-D, the olfactory-specific guanylyl cyclase is a marker for an apparently unique subset of olfactory neurons that also express phosphodiesterase type 2 (21). The number of mammalian guanylyl cyclase receptors is not known, but the recent work of Yu *et al.* (22), showing the existence of 29 or more guanylyl cyclases in *Caenorhabditis elegans*, many or most of which are expressed in distinct sensory neurons, raises the question of whether a significant number of orphan guanylyl cyclase receptors remain to be identified in the mammal. In 1990, Schulz *et al.* (12) obtained a PCR product from intestinal cDNA that was unique compared with the known mammalian cyclases described above. Within a short period of time, a partial length clone that included the cyclase catalytic and protein kinase-like domains was obtained from an intestinal cDNA library, but the full-length cDNA clone proved difficult to isolate, possibly due to mRNA secondary structure. Here, the sequence for the full-length cDNA clone is presented. The novel plasma membrane guanylyl cyclase receptor (GC-G) retains Cys residues conserved in the guanylyl cyclase-A receptor but is not activated by natriuretic peptides. The apparent receptor is expressed in highest amounts in lung, intestine, and skeletal muscle where it likely interacts with a unique class or subclass of ligands that signal through cGMP.

EXPERIMENTAL PROCEDURES

Cloning of GC-G—Poly(A)$^+$RNA prepared from small intestinal mucosa of the rat was reverse transcribed using an oligo(dT) primer and Superscript reverse transcriptase (Life Technologies, Inc.). First strand cDNA was used as a template for PCR with degenerate oligonucleotide primers based on conserved sequences within the catalytic domains of membrane and cytoplasmic forms of guanylyl cyclase (23). PCR products were cloned into M13 and sequenced. In addition to several known guanylyl cyclases, a unique sequence was amplified that was later shown to be GC-G. When the unique PCR product, designated SIM3,

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF024622.

§ Present address: Division of Clinical Pharmacology, Dept. of Medicine, Thomas Jefferson University, 1100 Walnut St., Philadelphia, PA 19107.

¶ Initially supported by a fellowship from Deutsche Forschungsgemeinschaft and to whom correspondence should be addressed: UTSWMC, 5323 Harry Hines Blvd., Dallas, TX 75235-9050. Fax: 214-648-5087; E-mail: bwedel@mednet.swmed.edu.

[1] The abbreviations used are: GC-A to -G, guanylyl cyclase-A to -G, respectively; ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide, CNP, C-type natriuretic peptide; STa, heat-stable enterotoxin from *E. coli*; *gucy2g*; gene for GC-G; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; IBMX, 3-isobutyl-1-methylxanthine.

was used to screen a small intestine cDNA library, a clone corresponding to GC-C, the heat-stable enterotoxin receptor, was obtained (12). Therefore, the sequence of SIM3 was extended using 3′-RACE (24). The sense guanylyl cyclase primer and an oligo(dT) antisense primer were used in the PCR, and a single product of 550 base pairs was obtained and sequenced. This PCR product encoded the 3′-end of GC-G and was subsequently used as a probe to screen the same library from which GC-C was obtained (12). Two partial length clones were obtained, both of which contained unspliced introns. Numerous cDNA libraries constructed in various manners were screened, and the longest cDNA clone obtained extended no further than about 300 base pairs 5′ of the putative transmembrane segment; this is the beginning of exon 6 based on other guanylyl cyclase sequences. A combination of 5′-RACE and genomic sequence information yielded exons 1–5. Predicted exon 1 was joined to exon 2 by splice overlap PCR and then assembled with exons 2–5 and the cDNA clone encoding exon 6 through the poly(A)$^+$ tail. The final construct was sequenced.

Northern Blots—For detection of GC-G mRNA, 5 μg of poly(A)$^+$ RNA was fractionated on a formaldehyde-1% agarose gel, then transferred to a nylon membrane. The membrane was probed with a radioactively labeled cDNA fragment corresponding to the 5′-end of GC-G (a part of the extracellular coding region; see Fig. 6*B*) or to part of the catalytic domain (see Fig. 6*C*). Following hybridization overnight at 42 °C, the membrane was washed at 60 °C in 0.5 × SSC, and mRNA corresponding to GC-G was visualized by autoradiography for 7 days at −80 °C (see Fig. 6*B*). For detection of GC-G mRNA in rat lung, the membrane was washed at 60 °C in 1 × saline/sodium phosphate/EDTA and exposed to a film for 3 days at −80 °C (see Fig. 6*C*). For detection of mRNA in H293 cells expressing GC-G, 2.5, 4.5, or 7.0 μg of total RNA were fractionated on a formaldehyde, 1% agarose gel and processed as described above except for washing conditions. The membrane was washed for 10 min at 65 °C with 2 × SSC, 0.1% SDS, for 20 min at 65 °C with 0.5 × SSC, 0.1% SDS and for 1 min at room temperature with 0.1 × SSC. The mRNA corresponding to GC-G was visualized using a phosphoimager (Fuji Biosystems).

GC-G Expression—The full-length GC-G cDNA was cloned into the mammalian expression vector pCMV5. For transient transfections, COS-7 cells were grown on 100-mm plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were transiently transfected using the DEAE-dextran method with 5 μg of plasmid (pCMV5, GC-G/pCMV5, or GC-A/pCMV5) per 100-mm plate. For stable expression, H293 cells were cotransfected with a pCMV5/GC-G construct and a pCMV5 construct containing a oubain-resistance gene using lipofection. H293 cells were grown in Opti-MEM I medium (Life Technologies, Inc.) for the first 24 h, which was then replaced with DMEM supplemented as described for COS-7 cells. For selection of ouabain-resistant clones, 0.5 μM ouabain was included in the medium. After several rounds of amplification, isolated oubain-resistant clones were tested for expression of GC-G by Western blot analysis.

Preparation of Membranes—COS-7 cells expressing GC-G were harvested 48 h after transfection; GC-G/H293 cells were grown to confluency before harvest. For preparation of membranes, cells were washed twice with 5 ml of ice-cold phosphate-buffered saline and scraped into 1 ml of ice-cold homogenization buffer (10% glycerol, 50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 μg/ml aprotinin). Cells were disrupted by sonication and centrifuged for 20 min at 2 °C and 100,000 × *g*. The membrane pellet was washed twice with homogenization buffer, and membranes were resuspended in 1 ml of homogenization buffer by sonication.

Guanylyl Cyclase Assay—Assays were performed in a total volume of 100 μl with 25 mM HEPES, pH 7.4, 50 mM NaCl, 0.25 mM IBMX, 0.1% bovine serum albumin, 10 mM NaN_3, 1 mM dithiothreitol, 100 μM GTP, 0.5 μCi/100 μl assay [α-^{32}P]GTP (NEN, 3000 Ci/mmol, 10 mCi/ml), and 5 mM $MnCl_2$ or $MgCl_2$ as well as 0.1% Triton X-100 as indicated. Reactions were started by the addition of membrane fraction to the assay mix and tubes were incubated for 10 min at 37 °C. Reactions were stopped with zinc acetate/sodium carbonate and radiolabeled [α-^{32}P]cGMP was isolated and quantified as described in Ref. 25.

Cyclic GMP Formation in Intact Cells—For determination of cGMP accumulation in COS-7 cells transiently expressing GC-G, 100-mm plates were washed twice with 2 ml of DMEM medium (see above, without additives) and incubated with DMEM containing 0.25 mM IBMX at 37 °C. After 10 min, this medium was exchanged with DMEM containing 0.25 mM IBMX without additives or with 1 μM ANP, 1 μM BNP, 1 μM CNP, or 1 μM STa and incubated for an additional 10 min. The reaction was stopped by addition of 2 ml of 1 N perchloric acid, and cGMP concentrations were estimated as described (25).

```
masrarsepplehrfyggaeshaghsslvltlfvvmlmtcleaAKLTVGFHAPW NISHPF    60
SVQRLGAGLQIAVDKLNSEPVGPG NLSWEFTYT NATCNAKESLAAFIDQVQREHISVLIG  120
PACPEAAEVIGLLASEWDIPLFDFVGQMTALEDHFWCDTCVTLVPPKQEIGTVLRESLQY  180
LGWEYIGVFGGSSAGSSWGEVNELWKAVEDELQLHFTITARVRYSSGHSDLLQEGLRSMS  240
SVARVIILICSSEDAKHILQAAEDLGLNSGEFVFLLLQQLEDSFWKEVLAEDKVTRFPKV  300
YESVFLIAPSTYGGSAGDDDFRKQVYQRLRRPPFQSSISSEDQVSPYSAYLHDALLLYAQ  360
TVEEMMKAEKDFRDGRQLISTLRADQVTLQGITGPVLLDAQGKRHMDYSVYALQKSG NGS  420
RFLPFLHYDSFQKVIRPWRDDL NAS GPHGSHPEYKPDCGFHEDLCRTKPPTGAGMTASVT  480
AVIP TVTLLVVASAAAITGLML WRLRGKVQNHPGDTWWQIHYDSITLLPQHKPSHRGTPM  540
SRCNVSNASTVKISADCGSFAKTHQDEELFYAPVGLYQGNHVALCYIGEEAEARIKKPTV  600
LREVWLMCELKHENIVPFFGVCTEPPNICIVTQYCKKGSLKDVLRNSDHEMDWIFKLSFV  660
YDIVNGMLFLHGSPLRSHGNLKPSNCLVDSHMQLKLAGFGLWEFKHGSTCRIYNQEATDH  720
SELYWTAPELLRLRELPWSGTPQGDVYSFAILLRDLIHQQAHGPFEDLEAAPEEIISCIK  780
DPRAPVPLRPSLLEDKGDERIVALVRACWAESPEQRPAFPSIKKTLREASPRGRVSILDS  840
MMGKLEMYASHLEEVVEERTCQLVAEKRKVEKLLSTMLPSFVGEQLIAGKSVEPEHFESV  900
TIFFSDIVGFTKLCSLSSPLQVVKLLNDLYSLFDHTIQTHDVYKVETIGDAYMVASGLPI  960
RNGAQHADEIATMSLHLLSVTTNFQIGHMPEERLKLRIGLHTGPVVAGVVGITMPRYCLF 1020
GDTVNMASRMESSSLPLRIHVSQSTARALLVAGGYHLQKRGTISVKGKGEQTTFWLTGKD 1080
GFAVPLPEFTEEEAKVPEIL                                          1100
```

FIG. 1. **The predicted amino acid sequence of GC-G.** Shown is the deduced amino acid sequence of GC-G starting with the putative initiation methionine (single letter code). Hydropathic analysis of the 1100 amino acid sequence predicts an amino-terminal hydrophobic signal peptide (*underlined* and in *lowercase letters*) with a cleavage site after residue Ala-43 (determined by the Sigseq program, The Rockefeller University). The putative transmembrane region is shown in *capital letters* and *underlined*, and five potential *N*-linked glycosylation sites (N*X*S or N*X*T) are marked in *bold*.

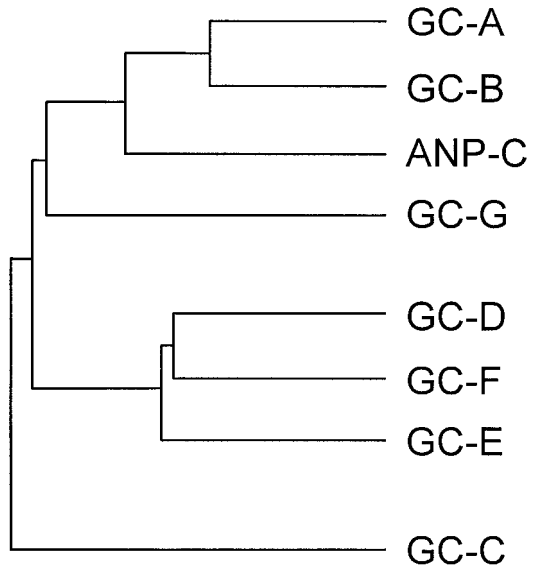

FIG. 2. **Dendrogram comparing mammalian guanylyl cyclases.** Shown is the similarity of the extracellular domains of guanylyl cyclases A-G (GC-A-G) and of the ANP-clearance (*ANP-C*) receptor. GC-G appears most closely related to the family of natriuretic peptide receptors (GC-A, GC-B, and ANP-C receptor). Analysis was performed using the DNAstar program Megalign, where the branch order is a function of structural similarity.

^{125}I-ANP Binding—Membranes from control or GC-G stably expressing H293 cells were prepared as described above. ^{125}I-ANP binding was basically assayed as described previously with the exception that What-

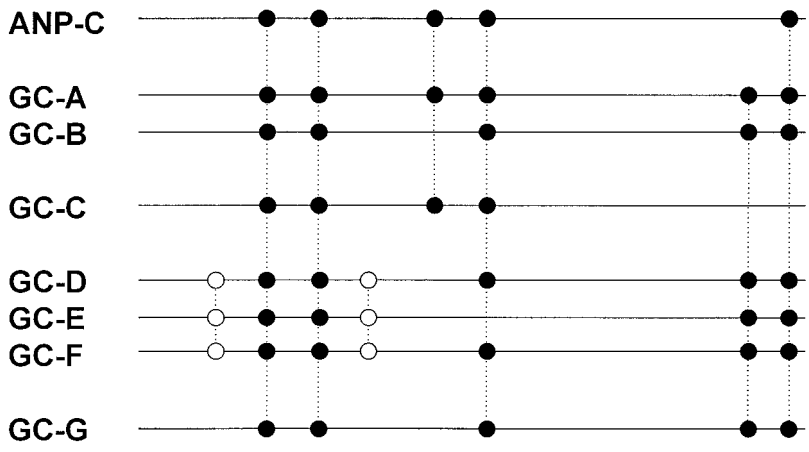

FIG. 3. **Cysteine residues in the GC-G extracellular domain are conserved in other guanylyl cyclases.** Shown schematically is the location of conserved Cys within the extracellular domain of the ANP-C receptor and guanylyl cyclases A-G. *Filled circles* indicate the location of conserved Cys, and *open circles* indicate Cys that are conserved within the neuronal family (*GC-D–F*). Two amino-terminal Cys are found within all receptors shown while another pair of Cys seems to be characteristic for the neuronal family of guanylyl cyclases. All five conserved Cys of GC-G align with the Cys of GC-B.

```
1    MPSLLVLTFSACVLLGWALLADCTGGGGSGGAGPGR----  ANP-CR
1    MASRARSE----------PPLEHRFYGGAESHAGHSSLVLT  GC-G

37   --GRRERERALPPQKIEVLVLLPQDDSYLFSLARVRPAIEY  ANP-CR
32   LFVVMLMTCLEAAKLTVGFHAPWNISHPFSVQRLGAGLQI  GC-G

75   ALRTVEGNATGRRLLPAGTRFQVAYEDSDCGNR-ALFSLV  ANP-CR
72   AVDKLNSEPVG----PGNLSWEFTYTNATCNAKESLAAFI  GC-G

114  DRVAAARGAKPDLILGPVCEYAAAPVARLASHWDLPMLSA  ANP-CR
108  DQVQREH----ISVLIGPACPEAAEVIGLLASEWDIPLFDF  GC-G

154  GALAAGFQHKDTEYSHLTRVAPSYAKMGEMMLALFRHHQW  ANP-CR
145  VGQMTALEDHFWCDTCVTLVPPK-QEIGTVLRESLQYLGW  GC-G

194  SRAVLVYSDDKLERNCFFTLEGVHEVFQ--EEGLH-----  ANP-CR
184  E-YIGVFGGSSAGSS----WGEVNELWKAVEDELQLHFTI  GC-G

227  TSAYNFDETKDLDLEDIVRHIQASERVVIMCASSDTIRGI  ANP-CR
219  TARVRYSSGHSDLLQEGLRSMSSVARVILICSSEDAKHI  GC-G

267  MLAAHRHGMTSGDYAFFNIELFNSSFYGDGSWKRGDKHDF  ANP-CR
259  LQAAEDLGLNSGEFVFLLLQQLEDSF-----WKEVLAEDK  GC-G

307  EAKQAYSSLQTITLLRTVKLEF-------EKFSMEVKSSVE  ANP-CR
294  VTR--FPKVYESVFLIAPS-TYGGSAGDDDFRKQVYQRLR  GC-G

341  KQ----GLSEEDYVNMFVEGFHDAILLYVLALREVLRAGY  ANP-CR
331  RPPFQSSISSEDQVSPYSAYLHDALLLYAQTVEEMMKAEK  GC-G

377  SKKDGGKIIQQTWNR--TFEGIAGQVSIDANGDRYGDFSV  ANP-CR
371  DFRDGRQLISTLRADQVTLQGITGPVLLDAQGKRHMDYSV  GC-G

415  IAMTDTEAGTQEVIGDYFGKEGRFEMRPNVKYPWGPLKLR  ANP-CR
411  YALQKSGNGSR-------FLPFLHYDSFQKVIRPWRD-DLN  GC-G

455  IDETRMVEHTNSSPCKASGGL-----EESAV           ANP-CR
444  ASGPHGSHPEYKPDCGFHEDLCRTKPPTGAG           GC-G
```

FIG. 4. **Alignment of the GC-G extracellular domain with the ANP clearance receptor (*ANP-CR*).** The alignment was performed using the DNAstar program Megalign. Amino acids (*single letter* code) are colored in groups as follows: H, K, and R, *light blue*; D, E, N, and Q, *dark blue*; A, G, P, S, and T, *green*; F, Y, and W, *magenta*; I, L, M, and V, *red*; and C, *yellow*.

man GF/C filters were used for filtration (26). Membranes were incubated with 200 pM ^{125}I-ANP in the absence or presence of 0.1 μM competing unlabeled ANP for 1 h at room temperature.

Western Blots—A synthetic peptide corresponding to the carboxyl-terminal decapeptide of GC-G (EEEAKVPEIL) was conjugated to PPD (2,5-diphenyl-1,3,4-oxadiazole, Serumstaaten Institute) using glutaraldehyde. The purified conjugate was used to raise a polyclonal antiserum in rabbits. For Western blotting, 25–50 μg of membrane protein of GC-G expressing COS-7 or H293 cells was separated on 8% sodium dodecyl sulfate-polyacrylamide gels. Proteins were electrophoretically transferred to polyvinylidene fluoride membranes, and nonspecific protein-binding sites of the membranes were blocked by incubation with TBST (Tris-buffered saline with Tween-20: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) containing 5% non-fat dry milk at room temperature for 1 h or at 4 °C overnight. Membranes were washed with TBST and incubated for 1 h at room temperature in a 1:1,000 dilution of primary antibody. Membranes were washed three times with TBST and then incubated with a 1:10,000 dilution of a horseradish-conjugated anti-rabbit antibody (Bio-Rad) in TBST. After washing the membranes three times with TBST, protein bands were detected using the enhanced chemiluminescence detection method (ECL, Amersham Corp.).

Chromosomal Localization—The chromosomal location of the gene encoding GC-G was determined by the use of a DNA panel derived from 94 progeny of an interspecific backcross (C57BL/6J × *Mus spretus*) of females with C57BL/6J males (Jackson BSB, Jackson Lab Backcross DNA Panel Map Service; Ref. 27). A sense primer (5′-TCTCAGAACT-

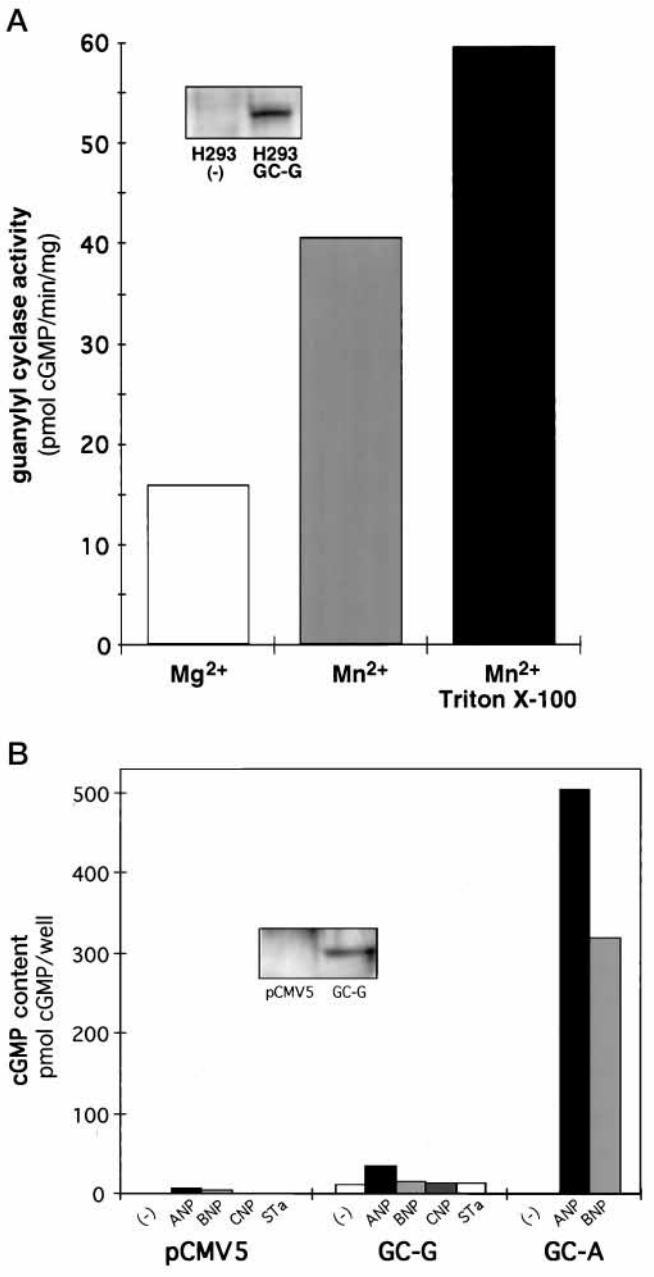

FIG. 5. **Guanylyl cyclase activity in cells overexpressing GC-G.** *A*, guanylyl cyclase activity in H293 cells stably expressing GC-G. Membranes of H293 cells expressing GC-G were assayed for guanylyl cyclase activity in the presence of 5 mM Mg^{2+}, 5 mM Mn^{2+}, or 5 mM Mn^{2+}, 0.1% Triton X-100. Guanylyl cyclase activity in membranes of mock transfected H293 cells was under the detection limit. *Inset*, Western blot showing expression of GC-G in H293 cells. *B*, cGMP content in COS-7 cells transiently expressing GC-G. COS-7 cells were transfected as described under "Experimental Procedures." Cells were incubated for 10 min at 37 °C in the absence or presence of 1 μM ANP, 1 μM BNP, 1 μM CNP, or 1 μM STa. As a control, cGMP content in GC-A-expressing cells in response to the natriuretic peptides ANP and BNP was determined. *Inset*, Western blot demonstrating expression of GC-G in COS-7 cells.

CAGAAGGAAAACTGC) in intron 1 (based on the rat genomic sequence) was used with an antisense primer in exon 2 (5′-ATGCTGGCAGTGATGTTGAAATGGA). A size polymorphism was detected (1 kb for *M. spretus* and 1.2 kb for C57BL/6). Backcross progeny DNA was typed using a standard PCR containing 125 ng of genomic DNA with 25 pmol of each primer in a total volume of 25 μl. DNA was denatured in an initial 5-min incubation at 95 °C followed by 5 min at 72 °C, during which time *Taq* polymerase was added. Thermal cycling was for 40 cycles (45 s at 94 °C, 45 s at 60 °C, 2 min at 72 °C) followed by a 20-min incubation at 72 °C. Reaction products were separated on an 0.8% agarose gel with parental samples for comparison.

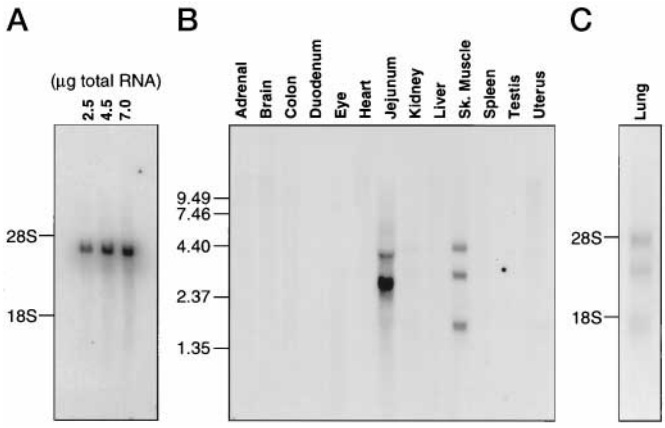

FIG. 6. **Tissue expression of GC-G mRNA.** *A*, total RNA (2.5, 4.5, or 7.0 μg) from H293 cells stably expressing GC-G was electrophoresed and blotted to nylon membranes. The blot was probed with a 5′-1.3 kb cDNA fragment of GC-G. The location of the 28 and 18 S RNA is shown. *B*, 5 μg of poly(A)$^+$ RNA from the indicated rat tissues was fractionated on a formaldehyde, 1% agarose gel and blotted to nylon membranes. The blot was probed as described above. Migration of RNA standards, in kilobases, is shown at *left*. *C*, 5 μg of poly(A)$^+$ RNA from rat lung was fractionated on a formaldehyde, 1% agarose gel and blotted to nylon membranes. The blot was probed with a cDNA fragment encoding the catalytic region of GC-G. Migration of the 28 and 18 S RNA is shown at *left*. Different sized mRNAs appear consistently evident in various tissues, suggesting alternative splicing.

RESULTS AND DISCUSSION

GC-G cDNA—The cDNA and predicted amino acid sequence for GC-G are given in Fig. 1. The open reading frame predicts a protein of 1100 amino acids containing a putative signal peptide of 43 amino acids. GC-G is similar to the other membrane forms of guanylyl cyclase (Fig. 2) in that a single transmembrane domain divides the protein approximately in half, separating an extracellular region from two prominent intracellular domains (protein kinase-like and cyclase catalytic). Of seven cysteine residues within the extracellular domain of GC-G, five align with five of six found in GC-A (Fig. 3). These cysteine residues are also conserved in other guanylyl cyclases (Fig. 3; Ref. 22). The ANP clearance receptor binds natriuretic peptides and contains an extracellular domain whose primary amino acid sequence is 30–40% identical to GC-A or GC-B (2, 3, 6, 28). Recent mapping of the disulfide bonding pattern of this receptor demonstrates that the first two Cys and second two Cys residues (from the amino terminus) form internal disulfide bonds (29, 30). The site of ANP binding has been mapped just to the amino-terminal side of the second disulfide loop (31), suggesting that this region is critical for ligand binding. Limited sequence information from genomic clones of GC-G also demonstrates that intron/exon boundaries are at the same positions in the GC-G gene as in the GC-A and the ANP clearance receptor genes (32, 33). The intron/exon boundaries are different in GC-C and in the sensory guanylyl cyclases (34),[2] and thus GC-G appears more closely related evolutionarily to the natriuretic peptide receptors than to other guanylyl cyclases (Fig. 2). A comparison of the extracellular domains of GC-G with the ANP clearance receptor, and a grouping of amino acids according to major chemical properties, shows that the two extracellular domains are more than 40% similar (Fig. 4).

Since the domain structure of GC-G is the same as that seen in guanylyl cyclases with known ligands, and Cys residues within the extracellular domain are conserved when compared

[2] S. Schulz, unpublished results.

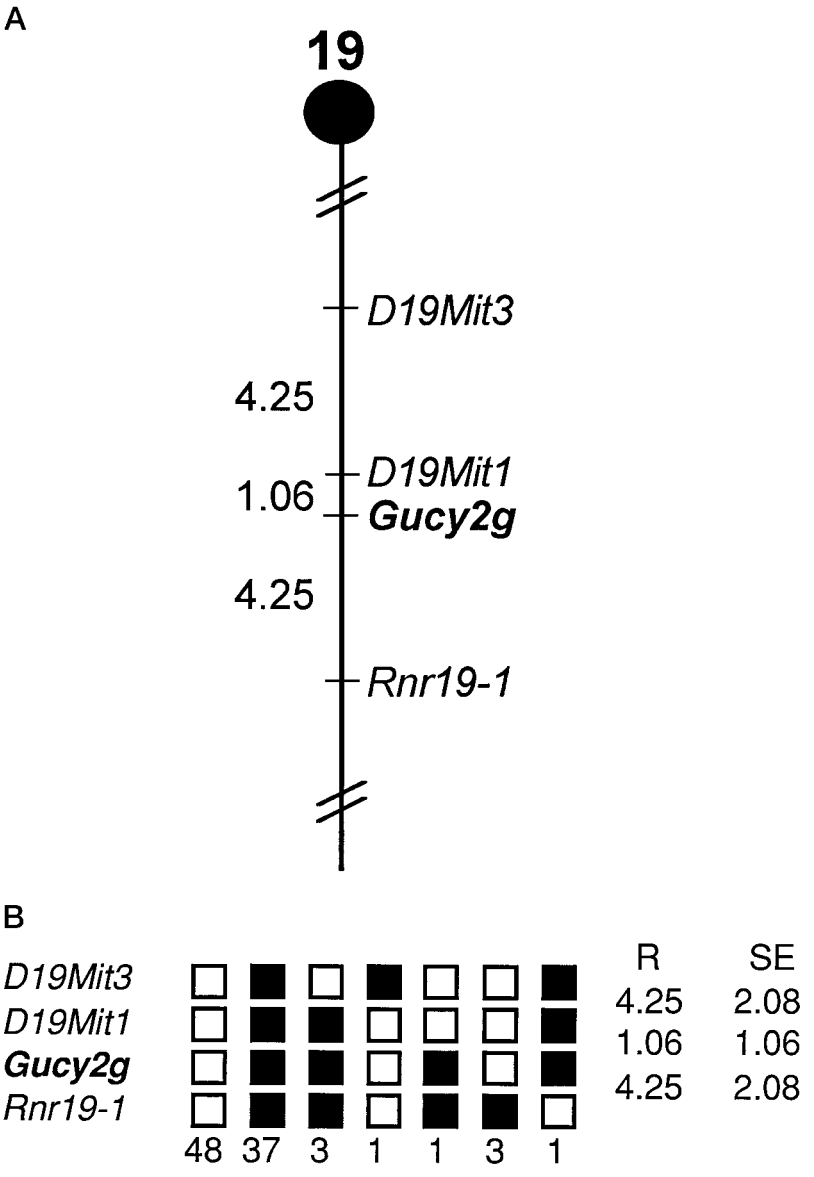

FIG. 7. **Chromosomal localization of the GC-G gene in mice.** *A*, shown is the localization of the GC-G gene (*gucy2g*) on mouse chromosome 19 relative to various markers. The GC-G gene was mapped by backcross analysis as described under "Experimental Procedures" (Jackson Laboratories). *B*, haplotype figure from The Jackson BSB backcross showing the distal end of chromosome 19 with loci linked to *gucy2g*. Loci are listed in order with the most proximal at the *top*. The *black boxes* represent the C57BL6/JEi allele, and the *white boxes* represent the SPRET/Ei allele. The number of animals with each haplotype is given at the *bottom* of each *column of boxes*. The percent recombination (*R*) between adjacent loci is given to the *right* of the figure, with the standard error (*S.E.*) for each R. Raw data from the Jackson Laboratory were obtained from the World Wide Web address http://www.jax.org/resources/documents/cmdata.

with cyclase receptors with known ligands, GC-G appears to represent a cyclase-coupled, orphan receptor. The high degree of similarity of GC-G with the natriuretic peptide receptor family within the putative ligand binding domain, as well as an apparent conservation of gene structure when compared with the GC-A or ANP clearance receptor genes, furthermore suggests that the putative ligand for GC-G may resemble a natriuretic peptide-like structure.

Expression of GC-G Activity—To confirm that GC-G encodes guanylyl cyclase activity and to facilitate searches for a putative ligand, the cDNA was expressed in either COS-7 or H293 cells. Cyclic GMP concentrations in either COS-7 or H293 cells were much higher than that seen when GC-A or GC-B are overproduced in the same cells (2, 35), suggesting that these cells do not effectively regulate GC-G activity (Fig. 5). Guanylyl cyclase activity was estimated in membranes prepared from H293 cells stably expressing GC-G (Fig. 5*A*). The addition of high concentrations (1 μM) of ANP, BNP, CNP, or STa had little, if any, effect on cell cGMP concentrations in COS-7 cells transiently expressing GC-G (Fig. 5*B*).

Natriuretic Peptide Binding—Since cGMP concentrations appeared poorly regulated by either COS-7 or H293 cells transfected with GC-G, direct binding assays were designed to determine whether GC-G might bind such a peptide with high affinity but fail to demonstrate regulation.

However, no detectable ^{125}I-ANP binding to the GC-G overproducing cells was detected (data not shown).

Tissue Expression of GC-G—Based on Northern hybridization, mRNA for GC-G is most prominent in lung, intestine, and skeletal muscle (Fig. 6, *B* and *C*). Various cell lines have been examined by reverse transcription-PCR and also contain detectable GC-G mRNA (data not shown). Multiple sized mRNAs were observed in the various tissues using a cDNA probe encoding the extracellular domain or the catalytic domain. A cDNA probe to the apparent intracellular region of GC-G also yielded three positive-hybridizing mRNA species (36). However, when the mRNA of H293 cells stably expressing GC-G was examined, only mRNA at the size of the predicted product was detected (Fig. 6*A*). Therefore, the multiple sized mRNAs in the various tissues likely reflects alternative splicing. That GC-G is in various peripheral tissues raises the possibility that the ligand for GC-G circulates. Its expression in skeletal muscle is particularly provocative since a role for cyclic GMP in muscle has been largely ignored. Conceivably, however, GC-G is a component of the smooth muscle vasculature in skeletal muscle as opposed to skeletal muscle itself. If so, then possibly

GC-G stimulation would lead to increased blood flow to the muscle in certain physiological states, such as in response to exercise. Our attempts to identify cells in which GC-G is expressed by *in situ* hybridization, however, have so far not been successful.

Chromosomal Localization—Based on these analyses, the gene for GC-G (*gucy2g*) maps to the distal region of mouse chromosome 19 and close to the anchor locus D19Mit1. This region is syntenic with human chromosome 10 q24–26. There do not appear to be genetic diseases in the human that have been mapped to the GC-G locus (Fig. 7).

GC-G represented a particularly difficult full-length cDNA to isolate. The recently reported sequence of a soluble guanylyl cyclase that contains a partial protein kinase homology domain (KsGC; Ref. 36) appears to represent a partial-length GC-G cDNA clone. The reported open reading frame for KsGC contains only a few differences from GC-G. However, the 3 base pairs upstream of the suggested initiation Met diverges from that of GC-G at a position identical to that of intron 12 of the GC-A gene (32). Therefore, the ksGC mRNA may contain an unspliced intron. That KsGC represents a splice variant of GC-G is not ruled out even though the proposed initiation Met does not coincide with an ideal Kozak consensus sequence (36).

Acknowledgments—We extend appreciation to Dr. David Goeddel and members of his research group, in particular Dr. Fred deSauvage, who provided considerable advice during the mid-phases of this work. We are grateful to Lynda Doolittle and Deborah E. Miller for excellent technical assistance. We also thank Lucy R. Rowe and Mary B. Barter (The Jackson Laboratory) for analysis of the BSB panel and advice in preparation of Fig. 7.

REFERENCES

1. Garbers, D. L., and Lowe, D. G. (1994) *J. Biol. Chem.* **269,** 30741–30744
2. Chinkers, M., Garbers, D. L., Chang, M.-S., Lowe, D. G., Chin, H., Goeddel, D. V., and Schulz, S. (1989) *Nature* **338,** 78–83
3. Lowe, D. G., Chang, M.-S., Hellmiss, R., Chen, E., Singh, S., Garbers, D. L., and Goeddel, D. V. (1989) *EMBO J.* **8,** 1377–1384
4. Lopez, M. J., Wong, S. K.-F., Kishimoto, I., Dubois, S., Mach, V., Friesen, J., Garbers, D. L., and Beuve, A. (1995) *Nature* **378,** 65–68
5. Kishimoto, I., Dubois, S. K., and Garbers, D. L. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6215–6219
6. Schulz, S., Singh, S., Bellet, R. A., Singh, G., Tubb, D. J., Chin, H., and Garbers, D. L. (1989) *Cell* **58,** 1155–1162
7. Koller, K. J., Lowe, D. G., Bennett, G. L., Minamino, N., Kangawa, K., Matsuo, H., and Goeddel, D. V. (1991) *Science* **252,** 120–123
8. Furuya, M., Aisaka, K., Miyazaki, T., Honbou, N., Kawashima, K., Ohno, T., Tanaka, N., Minamino, N., Kangawa, K., and Matsuo, H. (1993) *Biochem. Biophys. Res. Commun.* **193,** 248–253
9. Porter, J. G., Catalano, R., McEnroe, G., Lewicki, J. A., and Protter, A. A. (1992) *Am. J. Physiol.* **263,** C1001–C1006
10. Nakumura, M., Arakawa, N., Yoshida, H., Makita, S., and Hiramori, K. (1994) *Mol. Pharmacol.* **42,** 982–990
11. Drewett, J. G., Fendly, B. M., Garbers, D. L., and Lowe, D. G. (1995) *J. Biol. Chem.* **270,** 4668–4674
12. Schulz, S., Green, C. K., Yuen, P. S. T., and Garbers, D. L. (1990) *Cell* **63,** 941–948
13. Currie, M. G., Fok, K. F., Kato, J., Moore, R. J., Hamra, F. K., Duffin, K. L., and Smith, C. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 947–951
14. Hamra, F. K., Forte, L. R., Eber, S. L., Pidhorodeckyj, N. V., Krause, W. J., Freeman, R. H., Chin, D. T., Tompkins, J. A., Fok, K. F., and Smith, C. E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10464–10468
15. Forte, L. R., and Currie, M. G. (1995) *FASEB J.* **9,** 643–650
16. Schulz, S., Lopez, J. A., Kuhn, M., and Garbers, D. L. (1997) *J. Clin. Invest.*, **100,** 1590–1595
17. Yang, R.-B., Foster, D. C., Garbers, D. L., and Fülle, H.-J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 602–606
18. Shyjan, A. W., de Sauvage, F. J., Gillett, N. A., Goeddel, D. V., and Lowe, D. G. (1992) *Neuron* **9,** 727–737
19. Lowe, D. G., Dizhoor, A. M., Liu, K., Gu, Q., Spencer, M., Laura, R., Lu, L., and Hurley, J. B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5535–5539
20. Fülle, H.-J., Vassar, R., Foster, D. C., Yang, R.-Y., Axel, R., and Garbers, D. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3571–3575
21. Julifs, D. M., Fülle, H.-J., Zhao, A., Houslay, M. D., Garbers, D. L., and Beavo, J. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3388–3395
22. Yu, S., Avery, L., Baude, E., and Garbers, D. L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3384–3387
23. Yuen, P. S. T., Potter, L. R., and Garbers, D. L. (1990) *Biochemistry* **29,** 10872–10878
24. Frohman, M. A. (1993) *Methods Enzymol.* **218,** 340–356
25. Domino, S. E., Tubb, D. J., and Garbers, D. L. (1991) *Methods Enzymol.* **195,** 345–355
26. Wong, S. K. F., Ma, C-P., Foster, D. C., Chen, A.-Y., and Garbers, D. L. (1995) *J. Biol. Chem.* **270,** 30818–30822
27. Rowe, L. B., Nadeau, J. H., Turner, R., Frankel, W. N., Letts, V. A., Eppig, J. T., Ko, M. S., Thurston, S. J., Birkenmeier, E. H. (1994) *Mamm. Genome* **5,** 253–274
28. Fuller, F., Porter, J. G., Arfsten, A. E., Miller, J., Schilling, J. W., Scarborough, R. M., Lewicki, J. A., and Schenk, D. B. (1988) *J. Biol. Chem.* **263,** 9395–9401
29. Stults, J. T., O'Connell, K. L., Garcia, C., Wong, S., Engel, A. M., Garbers, D. L., and Lowe, D. G. (1994) *Biochemistry* **33,** 11372–11381
30. Itakura, M., Iwashina, M., Mizuno, T., Ito, T., Hagiwara, H., and Hirose, S. (1994) *J. Biol. Chem.* **269,** 8314–8318
31. McNicoll, N., Gagnon, J., Rondeau, J. J., Ong, H., and DeLean, A. (1996) *Biochemistry* **35,** 12950–12956
32. Yamaguchi, M., Rutledge, L. J., and Garbers, D. L. (1990) *J. Biol. Chem.* **265,** 20414–20420
33. Saheki, T., Mizuno, T., Iwata, T., Saito, Y., Nagasawa, T., Mizuno, K. U., Ito, F., Ito, T., Hagiwara, H., and Hirose, S. (1991) *J. Biol. Chem.* **266,** 11122–11125
34. Yang, R.-B., Fülle, H.-J., and Garbers, D. L. (1996) *Genomics* **31,** 367–372
35. Chinkers, M., and Garbers, D. L. (1989) *Science* **245,** 1392–1394
36. Kojima, M., Hisaki, K., Matsuo, H., and Kangawa, K. (1995) *Biochem. Biophys. Res. Commun.* **217,** 993–1000