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Tissue Distribution of the Human Soluble Guanylate Cyclases

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Soluble guanylate cyclase (sGC) is an important component of the NO signaling pathway. Human sGC isoforms α_1, α_2, and β_1 show differential mRNA tissue distributions. α_1 and β_1 are expressed in most tissues; however, the α_2 isoform shows a more restricted expression pattern with high levels in brain, placenta, spleen, and uterus only. Both α subunits exist as multiple transcripts whereas β_1 exists as a single message. This study reports for the first time the tissue distribution of human sGC message and demonstrates that sGC isoforms are nonuniformly expressed which may be useful if the enzyme is to be exploited as a therapeutic target.

Guanylate cyclase (GC) catalyses the conversion of guanosine-5′-triphosphate (GTP) to cyclic guanosine-3′,5′-monophosphate (cGMP) a second messenger in intracellular signaling cascades (for review see [1]). Two forms of GC enzymes have been characterized; a membrane form and a soluble form. Soluble GC (sGC) is a heterodimer consisting of α and β subunits [2] both of which are necessary for enzyme activity [3]. Both subunits possess a conserved catalytic domain at the C-terminus [4] and a heme binding domain in the N-terminus. Functional sGC requires intact N-termini of α and β subunits [5]. The free radical gas, nitric oxide (NO) can bind to the heme group of sGC resulting in the activation of the enzyme [6].

Several isoforms of sGC have been cloned and characterized. α_1 and β_1 subunits have been cloned from rat [4, 7], bovine [8, 9], human [10, 11], medaka fish [12] and *Drosophila* [13]. An α_2 isoform was originally identified in human fetal brain [14] and more recently in placenta [15] and a rat homologue has also been described [16]. Another isoform, β_2, has been found in rat kidney and liver [17]. Recently, RT-PCR has been used to amplify a 500-bp cDNA fragment from human heart that shows a high degree of identity to rat β_2 [18], however the full-length human β_2 sequence has yet to be characterized. Both α isoforms form a functional enzyme when coexpressed with the β_1 subunit, although the basal and NO stimulated rates are different with the $\alpha_1\beta_1$ heterodimer showing the greater activity [14]. Expression studies with the rat β_2 isoform have yielded an active enzyme with α_1 but again the dimer is less active than $\alpha_1\beta_1$ [19].

Although the different human isoforms of sGC have been known about for some time little has been done to determine their overall tissue distribution. The few studies that have been performed have looked at the relative distributions of α_1 and β_1 expression in the rat using either *in situ* hybridization or immunohistochemistry. Three studies of rat brain have located sGC mRNA predominantly in striatum, the olfactory system and layers II and III of the cerebral cortex [20–22]. Kummer *et al.* investigated rat and guinea pig sensory ganglia in immunohistochemical studies and showed the presence of α_1 and α_2 at the protein level [23]. Immunohistochemistry and immunofluorescence demonstrated the expression of α_1 and β_2 isoforms in rat kidney but no specific immunoreactivity with antibodies to β_1 or α_2 was observed [24]. There is very little information regarding expression of human isoforms of sGC. Zabel *et al.* demonstrated the presence of α_1 and β_1 protein in brain and lung but not in kidney, liver or pancreas [11].

Extensive research has shown that the NO-sGC-cGMP signaling pathway is widespread in mammalian tissues and important in mediating numerous physiological processes including vascular and non-vascular smooth muscle relaxation, peripheral and central neurotransmission, platelet reactivity and phototransduction. This makes sGC an attractive therapeutic target for a large range of conditions including angina, myocardial infarction, tissue hypoxia, glaucoma, thrombosis and septic shock. An understanding of human sGC

Abbreviations used: sGC, soluble guanylate cyclase; NO, nitric oxide; UTR, untranslated region.

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isoform expression and tissue distribution would be invaluable in the advancement of sGC as a target and subsequent drug design. In this study we have used degenerate PCR primers designed against the conserved catalytic domain of GC to try and identify novel sGC isoforms. Human RNA dot blots and northern blots were used to determine the relative distribution of the known human isoforms α_1, α_2, and β_1.

MATERIALS AND METHODS

PCR primers were designed against the conserved motifs, VYKVET and MPRYC, that have been identified in the catalytic domain of all soluble and particulate GCs. Degeneracy was designed into the primers such that they would amplify all the known subunits: forward primer: 5′-TGTBTAYAAGGTRGARAC-3′, reverse primer: 5′-ARRCARTANCKDGGCAT-3′. 250 ng human fetal brain RNA (Clontech) was reversed transcribed using SUPERSCRIPT II reverse transcriptase (Life Technologies) and 150 ng random primers (Promega). One microliter of human fetal brain cDNA was used as a template in PCR with 4 ng each primer and 1.25 u *Taq* DNA polymerase (Life Technologies) for 30 cycles (95°C 1 min, 55°C 1 min, 72°C 2 min). PCR products of the expected size for GC were cloned using the TA cloning kit (Invitrogen). Over 100 hundred clones were sequenced using an ABI 377 automatic sequencer (Perkin–Elmer).

Specific cDNA probes for human sGC α_1, α_2, and β_1 and rat sGC α_1 were made as detailed above with the following changes. 250 ng human adult brain RNA or rat brain RNA (Clontech) were used for the RT step. Specific primers were designed against the published human sequences for α_1, α_2, and β_1. α_1 forward primer: 5′-CTGATTTTCCCAGAGTTTGAA-3′ (bases 764–784), reverse primer: 5′-CTTAGGGAAGAAGTAGTAA-3′ (bases 1147–1129). α_2 forward primer: 5′-TTTCTTCGTCGAGAATA-3′ (bases 2519–2535), reverse primer: 5′- TAGTTCTGAAAGGGGACCCA-3′ (bases 2872–2853). β_1 forward primer: 5′-GCTGCAAGCAAAGTCCTC-3′ (bases 248–265), reverse primer: 5′-GATGCGTGATTCCTGGGT-3′ (bases 712–695). 1 μl human adult brain cDNA was used as a template in PCR for 30 cycles (95°C 1 min, 55°C 1 min, 72°C 2 min). 1 μl rat brain cDNA (Clontech) was used with the α_1 primers in a PCR to generate a rat specific α_1 probe. The PCR fragments were cloned using the TA cloning kit and sequenced using an ABI 377 automatic sequencer.

The cDNA probes (50 ng) were labeled using a random primed DNA labeling kit (Boehringer-Mannheim) and [α-^{32}P]dCTP (Amersham International). Nonincorporated dNTPs were removed using Sephadex NICK columns (Pharmacia). Human MTN Blots and an human RNA Master Blot were purchased from Clontech. Human RNA Master Blot was hybridized with the probes for α_1, β_1 and ubiquitin as control. The MTN Blots were hybridized with the probes for all three isoforms and also an actin control probe. A rat MTN blot was hybridized with a rat specific α_1 probe and an actin probe. All blots were hybridized and washed using conditions recommended by the manufacturer. Washed blots were exposed to Kodak Biomax MS film (Anachem) at −70°C for various lengths of time and then developed. Quantitation of autoradiographs was performed using a scanning laser densitometer and Molecular Analyst software (Bio-Rad). Densitometer results were normalized against either the ubiquitin or actin control and expressed as a percentage of the maximum.

RESULTS

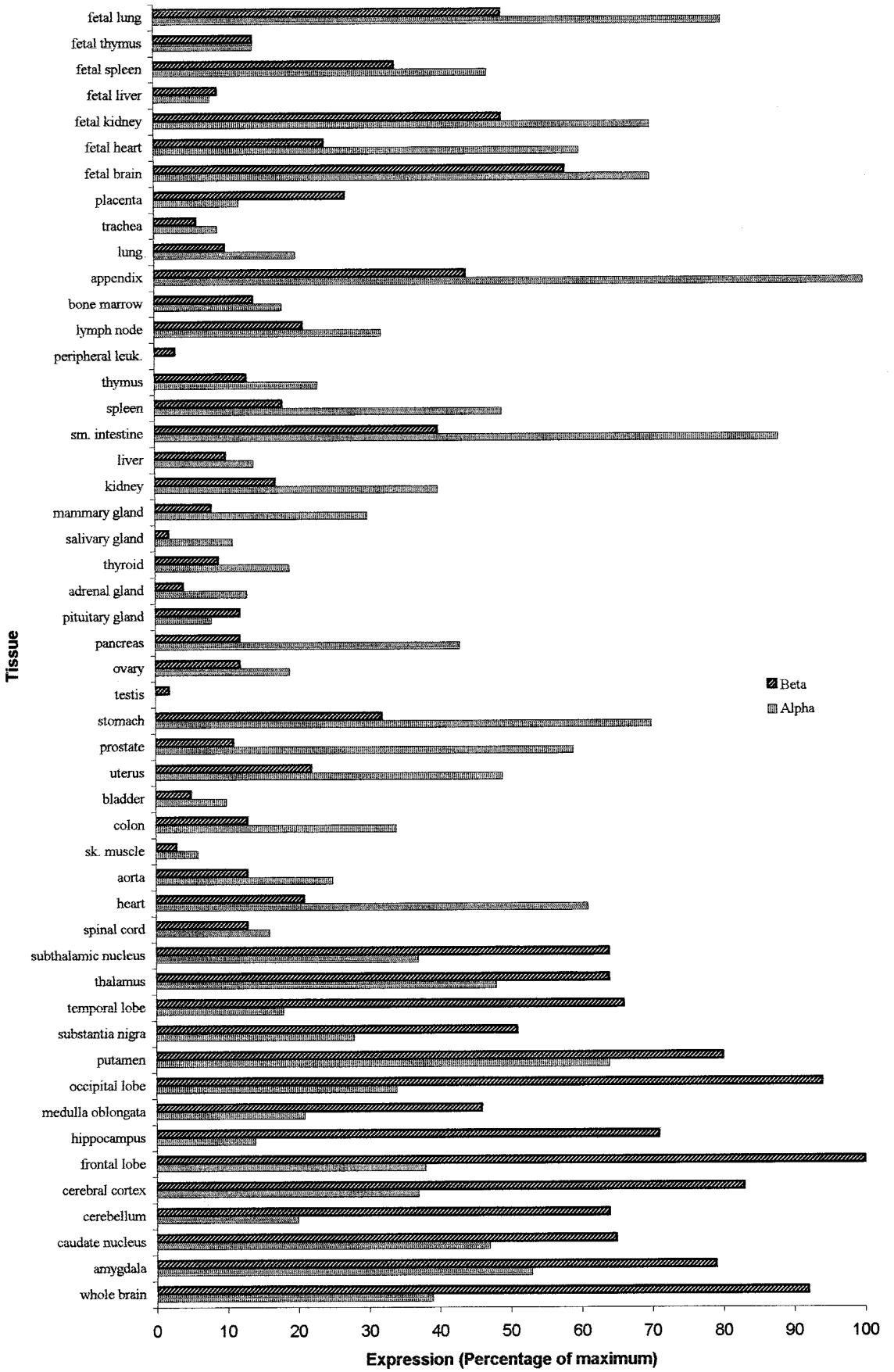
To ensure that we would study the tissue distribution of all major sGC isoforms PCR using degenerate primers against conserved motifs within the catalytic domain of all GCs was performed to try and amplify any previously unidentified human sGC isoforms. More than 100 clones were sequenced and the sGC isoforms α_1 and β_1 were amplified as well as particulate GCs and a number of GTP binding proteins such as Rab3a, however, no novel sGC-like sequences were identified.

The probes used for the study of α_1, α_2 and β_1 were carefully designed such that they were specific for each isoform. The α_1 and β_1 probes were derived from regions at the N-termini where the subunits show the least similarity. The α_2 probe was designed against the 3′ untranslated region.

The overall human tissue distribution of sGC α_1 and β_1 is shown in Fig. 1. The results are shown as a percentage of the maximum expression after correction for loading differences. In all the regions of the brain investigated, pituitary gland and placenta β_1 mRNA expression is greater than α_1. Most of the other tissues studied showed greater α_1 expression compared to β_1 especially heart, prostate, small intestine and appendix. Some of the tissues displayed very low levels of expression of both subunits including skeletal muscle, bladder, testis and peripheral leukocytes. In contrast to the adult tissue, fetal brain showed similar levels of α_1 and β_1 message. Both adult and fetal heart displayed more α_1 than β_1. Fetal lung showed greater sGC expression than adult lung.

Northern blots of some of the tissues were used to verify the results seen with the RNA dot blot. Figure 2 shows the results obtained with probes for α_1 (A), β_1 (B), and α_2 (C). The α_1 probe detected three different transcripts of approximately 3.5, 4, and 9 kb. This unexpected result was confirmed using a probe complementary to the 3′ untranslated region of human α_1. The same hybridization pattern was observed (result not shown). The distribution pattern of the three transcripts show a similar tissue distribution and complement the results seen with the dot blot. Very strong levels of α_1 message were detectable in heart with less in skeletal muscle, thymus, testis and leukocytes. However, the high level of α_1 message seen in small intestine suggested by the dot blot was not borne out by the northern blot. Human α_2 also exists as multiple transcripts of approximately 5, 6.5, and $>$9 kb and again the three messages show similar abundance in the selected tissues. The most notable differences between human α_1 and α_2 distribution are in the heart, kidney, prostate and colon where α_1 expression is greater than α_2. Expression of α_2 mRNA is high in brain, placenta, spleen and uterus relative to the other tissues examined.

The message for human β_1 can be identified as a single transcript of 3.5 kb and the distribution pattern is similar in the northern blots and dot blot. Overall the pattern of β_1 expression is similar to α_1. Very high levels of β_1, compared to other tissues, are observed in those tissues that express both α subunits such as the brain, placenta and uterus.


fetal lung
fetal thymus
fetal spleen
fetal liver
fetal kidney
fetal heart
fetal brain
placenta
trachea
lung.
appendix
bone marrow
lymph node
peripheral leuk.
thymus
spleen
sm. intestine
liver
kidney
mammary gland
salivary gland
thyroid
adrenal gland
pituitary gland
pancreas
ovary
testis
stomach
prostate
uterus
bladder
colon
sk. muscle
aorta
heart
spinal cord
subthalamic nucleus
thalamus
temporal lobe
substantia nigra
putamen
occipital lobe
medulla oblongata
hippocampus
frontal lobe
cerebral cortex
cerebellum
caudate nucleus
amygdala
whole brain
Tissue
Beta
Alpha
0
10
20
30
40
50
60
70
80
90
100
Expression (Percentage of maximum)

To determine if there were species specific differences in the α_1 subunit mRNA structures a rat northern blot was hybridized with a probe complementary to rat α_1 sequence. The rat probe was designed against the same part of the α_1 sequence as the human probe. The resulting autoradiograph (Fig. 3) shows that rat α_1 exists as a single transcript of 5.5 kb unlike our results for human α_1. The pattern of rat α_1 tissue expression is similar to the human with high levels in the heart and lower expression in skeletal muscle and testis.

DISCUSSION

Little is known about the human isoforms of sGC. Most of the characterization studies have used either the bovine or rat forms of the enzyme. Our study of human sGC involved a search for novel isoforms and a detailed analysis of the tissue distribution of the known isoforms.

Using degenerate primers designed against conserved motifs we sequenced over a hundred clones. Both soluble and particulate GCs were amplified by this strategy but no novel sGC-like sequences were identified. In 1997, Yu *et al.* published data that showed that at least 29 guanylate cyclase sequences existed in *C. elegans* [25]. Of these sequences only three were labeled as putative sGCs, the rest being particulate forms of the enzyme. A novel β subunit of sGC has recently been described in the insect *Manduca sexta* but although this isoform shows highest similarity to rat sGC β_1 it has many features that distinguish it from other sGCs [26]. These include the fact that it does not need to form heterodimers to form an active enzyme and its activity is only weakly enhanced by NO. It also has an additional 315 amino acids at the C-terminus. Thus this subunit may represent a novel member of the guanylate cyclase family rather than a novel isoform of sGC. These data suggest that sGC may not be an extended family but may be limited to the four isoforms already described. It was for this reason that we concentrated our efforts on determining the tissue distribution of the three known human isoforms α_1, α_2 and β_1.

Our data clearly show that there are discrepancies at the mRNA level between α_1 and β_1 expression in the human brain. Zabel *et al.* suggested that because the relative concentrations of α_1 and β_1 were different in human brain and lung that complementary sGC subunits are not necessarily expressed in a 1:1 ratio [11]. Our results suggest an alternative explanation is that the additional presence of α_2 in the brain, which can also form a dimer with β_1, allows a 1:1 ratio of complementary subunits to be expressed.

Another tissue to show higher β_1 than α_1 expression is the placenta where the existence of an $\alpha_2\beta_1$ dimer has recently been described [15]. Previous work has demonstrated that the activity of sGC isolated from human placenta is lower following stimulation by NO than the enzyme isolated from lung [27]. This may be explained by a different activity of the $\alpha_2\beta_1$ heterodimer. The remaining tissues investigated in this study express more α_1 than β_1.

This study has shown that the tissue expression of α_1 and α_2 is not identical. Out of all the tissues investigated, only the brain, placenta, pancreas, spleen and uterus have detectable levels of α_2. Notably the heart which has very high levels of α_1 mRNA does not express α_2. Thus expression of α_2 seems to be restricted to certain tissues.

It should be remembered that all the tissues analyzed in this study are composed of multiple cell types. It is possible that individual cell types may express specific sGC isoforms. For example, in the rat kidney, immunohistochemistry has shown the presence of α_1 in glomerular podocytes and β_2 in the principal cells of the collecting duct [24].

The transition from fetal to neonatal life involves a marked decline in pulmonary vascular resistance which is modulated in part by endothelium derived NO. Since NO binds to and activates sGC it is of no surprise to find greater levels of the enzyme in fetal lung compared to adult.

The existence of multiple transcripts has not been reported before for sGC but they are common in mammalian and viral genomes. Multiple transcripts may arise from a variety of mechanisms including alternative splicing, multiple transcription start points and by multiple poly-A signals. Caesin kinase II exists as a heterotetramer of 2 α and 2 β subunits. The β subunit has a single message but α has multiple transcripts similar to the results reported here for sGC [28].

Two other enzymes involved in the NO signaling pathway are known to exist as multiple transcripts. Multiple promoters are responsible for various species of human nNOS mRNA. Identical proteins are produced but the differences in the 5′ untranslated region (UTR) could affect mRNA processing, localization, stability and translation efficiency [29]. Three different forms of cDNA exist for human GTP cyclohydrolase I, however, functional protein is only encoded by the longest reading frame [30]. It is not known whether all three transcripts for sGC α_1 and α_2 are translated. It is

FIG. 1. Tissue distribution of human sGC α_1 and β_1. A RNA dot blot (Clontech) was hybridized with probes specific for human α_1, β_1 and ubiquitin and exposed to X-ray film. Results were quantified using laser densitometry, normalized against ubiquitin and expressed as a percentage of the maximum expression for each isoform.

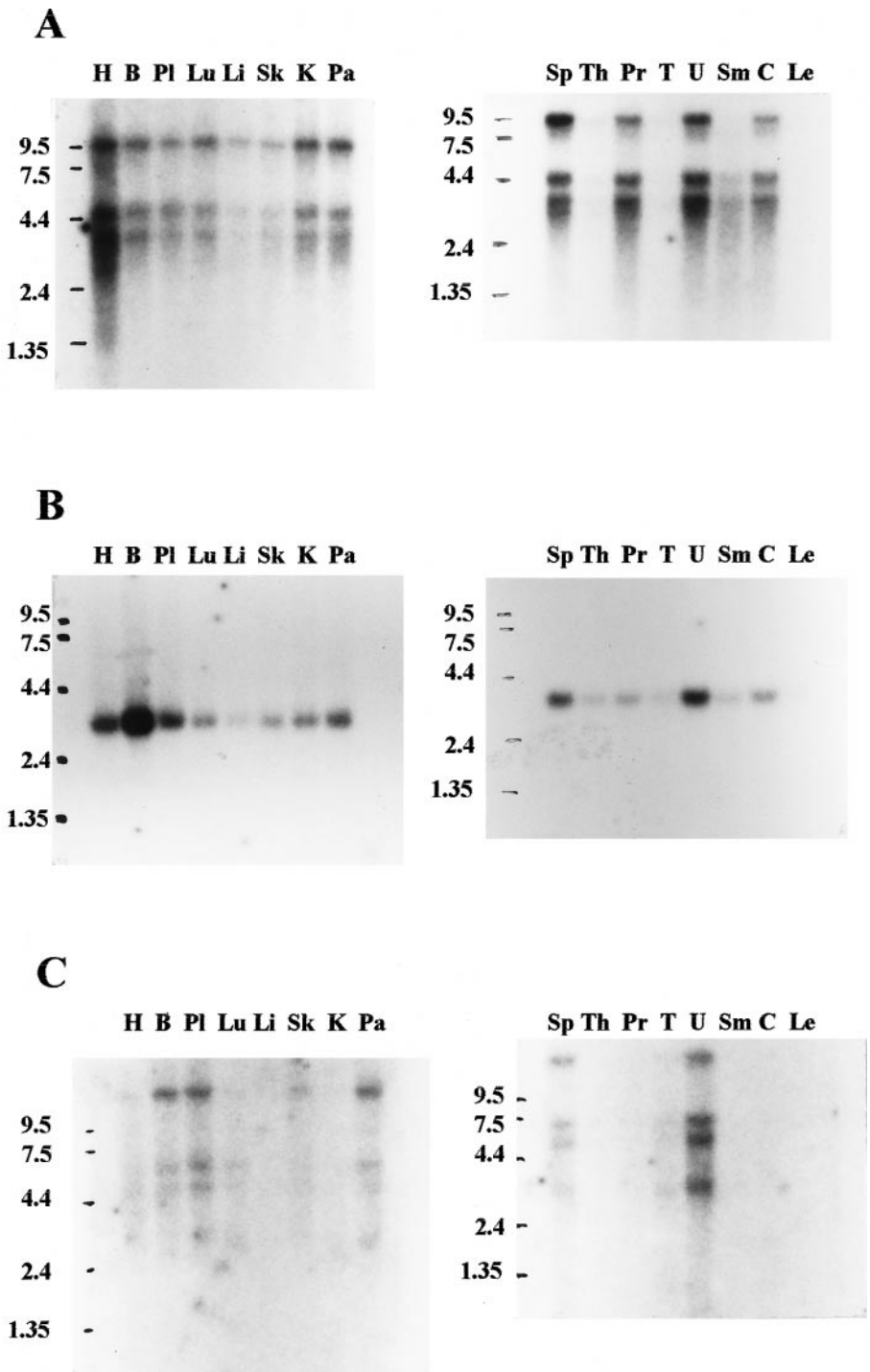


FIG. 2. Northern blots of human sGC α_1, β_1 and α_2. Northern blots were hybridized with probes specific for human α_1 (A), β_1 (B) and α_2 (C) and exposed to x-ray film. Blots were hybridized with an actin probe to check for equal loading. The tissues investigated were: (H) heart; (B) brain; (Pl) placenta; (Lu) lung; (Li) liver; (Sk) skeletal muscle; (K) kidney; (Pa) pancreas; (Sp) spleen; (Th) thymus; (Pr) prostate; (T) testis; (U) uterus; (Sm) small intestine; (C) colon; (Le) leukocytes. Marker sizes are indicated (kb).

possible that only a single mRNA species is translated into a functional protein similar to human GTP cyclohydrolase I.

A probe against sGC α_1 detects a single message in rat tissues but three differently sized messages in human. Species differences such as these have been observed before for other genes. The mouse opsin gene exists as 5 major transcripts but in the rat there are 4, human 3, and bovine 1. For this gene the utilization of different poly-A signals results in 3′ heterogeneity [31].

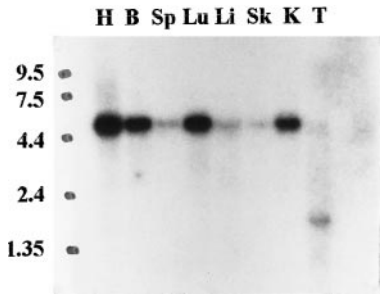


FIG. 3. Northern blot of rat sGC α_1. The blot was hybridized with a probe specific for rat α_1 and exposed to x-ray film. An actin probe was used to check for equal loading. Tissues investigated were: (H) heart; (B) brain; (Sp) spleen; (Lu) lung; (Li) liver; (Sk) skeletal muscle; (K) kidney; (T) testis. Marker sizes are indicated (kb).

The sGC α_1 and α_2 transcripts do not appear to be tissue specific. The significance of these multiple α transcripts will not become apparent until the gene structures for sGC α_1 and α_2 have been determined. This will also indicate whether sGC α_1 and α_2 are under any transcriptional control by tissue or cell type specific promoters. This may reflect a way of regulating sGC activity by tightly controlling the transcription of the α subunits.

Modulators of sGC have potential as therapeutic agents and isoform specific compounds may have benefits over existing compounds. This study has highlighted significant differences in the mRNA expression of the human α_1, α_2 and β_1 isoforms of sGC. It has also identified heterogeneity at the mRNA level for the α isoforms. This report shows a pattern of human sGC tissue expression which may indicate potential clinical uses and will be important if the enzyme is to be fully exploited as a therapeutic target especially in the CNS and cardiovascular systems.

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