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Genetics

G-utrophin, the autosomal homologue of dystrophin Dp116, is expressed in sensory ganglia and brain

(Duchenne muscular dystrophy/alternative transcript/mouse development/neuron)

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ABSTRACT **The utrophin gene is closely related to the dystrophin gene in both sequence and genomic structure. The Duchenne muscular dystrophy (*DMD*) locus encodes three 14-kb dystrophin transcripts in addition to several smaller isoforms, one of which, Dp116, is specific to peripheral nerve. We describe here the corresponding 5.5-kb mRNA from the utrophin locus. This transcript, designated G-utrophin, is of particular interest because it is specifically expressed in the adult mouse brain and appears to be the predominant utrophin transcript in this tissue. G-utrophin is expressed in brain sites generally different from the regions expressing β-dystroglycan. During mouse embryogenesis G-utrophin is also seen in the developing sensory ganglia. Our data confirm the close evolutionary relationships between the *DMD* and utrophin loci; however, the functions for the corresponding proteins probably differ.**

Utrophin is a high-molecular-weight protein that has extensive primary sequence similarity to the muscle protein dystrophin (1). In normal, adult skeletal muscle utrophin is found at the neuromuscular junction, whereas dystrophin predominates at the sarcolemma, where it is a component of an oligomeric membrane-spanning complex, the dystrophin-associated glycoprotein complex (DGC) (for review, see ref. 2). In dystrophin-deficient muscle, utrophin copurifies with components of the DGC, indicating that utrophin can also bind to this complex (3). At the neuromuscular junction, utrophin is found at the crests of the postsynaptic folds precisely colocalized with agrin-induced acetylcholine receptor clusters (4–6). Recent studies have shown that α-dystroglycan, a component of the DGC, is a functional agrin receptor (5–7). Taken together, these findings suggest that in skeletal muscle utrophin links the actin-based cytoskeleton of the neuromuscular junction with the basal lamina via α-dystroglycan.

Utrophin is found in many other tissues in addition to muscle; for example, it occurs at significant levels in smooth muscle and is relatively abundant in adult lung and intestine (8, 9). In the brain utrophin is found in the astroglia surrounding the brain capillaries (10), and it is also expressed in cultured schwannoma and glial cells (8, 11). During early development utrophin mRNA is found at high levels in the neural tube and is abundant in a subset of neural crest-derived tissues—in particular, the sensory ganglia. Later in development utrophin is also expressed in a variety of other sites—such as the tendon primordia of the developing digits, the pituitary gland, thyroid gland, adrenal gland, gut and bladder mucosae, ossifying cartilages, cardiac muscle, and the follicles of the developing vibrissae (12). Little is known of the subcellular localization or function of utrophin in these various embryonic and adult tissues. It is of interest that α-dystroglycan and some other components of the DGC have a wider tissue distribution than dystrophin; thus utrophin may be implicated in membrane glycoprotein complexes with these components in sites other than muscle.

Dystrophin is largely confined to skeletal, cardiac, and smooth muscle and to the brain; expression at these sites is regulated by at least five dystrophin promoters with distinct cell-type specificities. In the brain there are two dystrophin forms: C-dystrophin, expressed in cortical neurons, and P-dystrophin, expressed in cerebellar Purkinje cells (for review, see ref. 13). These dystrophins are transcribed from two promoters that flank the muscle promoter and encode 400-kDa proteins that differ from muscle dystrophin by only a few amino acids at the N terminus. Two other proteins are encoded from two promoters in the distal part of the *DMD* gene (for review, see ref. 14). Apo-dystrophin-1, or Dp71, is a 71- to 77-kDa protein found in glial cells in the developing embryo and in adult brain (15–18). Apo-dystrophin-2, or Dp116, is a 116-kDa protein found in peripheral nerve (19) and in the embryonic brain (18).

The close structural/evolutionary relationship between utrophin and dystrophin suggests that utrophin may also be characterized by transcription from multiple tissue-specific promoters. In this paper we describe the isolation and characterization of a transcript from the utrophin locus that is transcribed from a promoter in the distal part of the utrophin gene and shows widespread expression in the adult brain and in sensory ganglia.¶ Because this transcript is first detected in ganglia, we have adopted the nomenclature first suggested by Górecki *et al.* (20) and called this product G-(for ganglia) utrophin.

MATERIALS AND METHODS

RNA Extraction, Northern Blotting, and RNase Protection Assay. RNA was extracted from mouse tissue or cultured cells by the method of Chomczynski and Sacchi (21). mRNA was prepared using Oligotex (Qiagen, Chatsworth, CA). Northern blots were prepared as described (15). RNase protection assays were done on 20 μg of total RNA using an RPAII kit (Ambion, Austin, TX). Antisense cRNA probes were labeled with [^{32}P]CTP using SP6 RNA polymerase and purified by column chromatography. The probe used in this experiment encompassed nt 246–430 of the G-utrophin sequence and was subcloned into the *Eco*RI site of pGEM-7Zf(+). Protected

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Abbreviations: DGC, dystrophin-associated glycoprotein complex; 3′UTR and 5′UTR, 3′ and 5′ untranslated region, respectively.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. X83506).

ragments were separated on a 6% denaturing polyacrylamide gel.

cDNA Library Construction. A cDNA library was made from 5 μg of adult mouse brain mRNA by the method of Gubler and Hoffman (22). Adapted cDNA was ligated into the *Bst*XI sites of pcDNAII (Invitrogen). The cDNA library was plated and screened by using standard procedures.

Sequence Analysis. Nested deletions of the cDNA clones were sequenced with Sequenase (United States Biochemical). Sequences were manipulated with the Genetics Computer Group (Madison, WI) version 7.3 software package.

***In Situ* Hybridization.** RNA *in situ* hybridization analyses were done by using either oligonucleotide probes or cRNA probes. Oligonucleotide hybridizations were done as described (20). Two oligonucleotides were used in this study: G-UT, 5′-TGTAACAAAACCACAACTTTCTGCAGAGACGT-TCTCACCACGGCC, nt 245–289, complementary to the unique 5′-end of G-utrophin, and UT, 5′-TCAGTGT-CAAGTGAGTAGCTCAATGCAGAATGCTGTG-GGGAAGCC, nt 2966–3010, complementary to the 3′-end common to the full-length utrophin and G-utrophin. *In situ* hybridizations with cRNA probes were done as described by Schofield *et al.* (12). The probes used were as follows: a 340-bp *Eco*RI/*Kpn* I subclone of au#3; Gu5′ E/K, consisting of the unique 5′ sequence of G-utrophin; and a 1.0-kb *Hin*dIII/*Eco*RI subclone of Bml102, u3′UTR H/E, containing the last kilobase of the mouse utrophin 3′ untranslated region (3′UTR) as described (12). Sense and antisense cRNA probes were hybridized to adjacent sections.

RESULTS

Identification and Cloning of G-Utrophin. G-utrophin was identified as a prominent 5.5-kb transcript on Northern blots of adult mouse brain mRNA (Fig. 1) hybridized with a probe that encodes the C-terminal half of rat utrophin (R.A.Z., D.J.B., and K.E.D., unpublished work). The 5.5-kb mRNA was not detected in mRNA from early embryonic mouse brain, BC3H-1 cells [an abundant source of utrophin mRNA (11)], or monkey COS-7 cells. A cDNA library, constructed from adult mouse brain mRNA, was screened with the C-terminal utrophin probe. Three positively hybridizing clones, au#1 (5.0 kb), au#2 (1.1 kb), and au#3 (5.5 kb), were picked. Clone au#3 was considered likely to be a full-length cDNA clone because it was

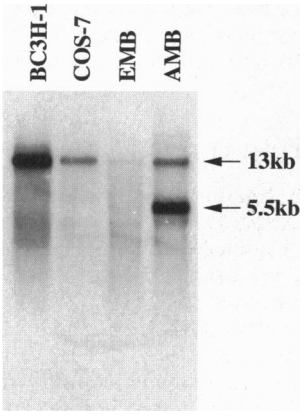

FIG. 1. Northern blot hybridized with the rat utrophin cDNA clone, a219EcoRI 5.0 (Fig. 2*A*), showing the utrophin and G-utrophin transcripts. BC3H-1, mouse BC3H-1 smooth muscle-like cell line from a mouse brain tumor; COS-7, monkey kidney COS-7 cells; EMB, embryonic mouse brain; AMB, adult mouse brain. The 13-kb utrophin transcript is present in all samples but is only just detectable in embryonic mouse brain mRNA. The 5.5-kb G-utrophin transcript is detected only in adult mouse brain mRNA.

the same size as the G-utrophin mRNA determined by Northern blotting; this was confirmed by sequence analysis.

Conceptual translation of the G-utrophin sequence, calculated from the first methionine of the open reading frame (Fig. 2*A*), predicts a protein of 113 kDa in the same reading frame as utrophin (Fig. 2*B*). There are several noteworthy features of the G-utrophin sequence. (*i*) The 5′ untranslated region (5′UTR) of G-utrophin includes five ATG sequences out-of-frame with each other, whereas the unique open reading frame has four in-frame ATG codons preceding the common utrophin sequence (Fig. 2*A*). The Kozak consensus sequences of these potential initiation codons are all poor and may indicate that the G-utrophin transcript is poorly translated (23). (*ii*) The point at which G-utrophin differs from utrophin is the same as that in the dystrophin gene where apo-dystrophin-2 (Dp116) differs from dystrophin—that is, between exons 55 and 56 (18, 19). (*iii*) The only obvious similarity between the unique N-terminal sequences of G-utrophin and apo-dystrophin-2 is four amino acids, SPRY in G-utrophin (boxed in Fig. 2*A*) and SPRF in apo-dystrophin-2 (18), both of which are potential phosphorylation sites for protein kinase C (24).

The unique 5′ sequence of G-utrophin and the deduced N-terminal amino acid sequence have no significant similarity to any sequence listed in the GenBank data base. Comparison of the region of overlap between mouse G-utrophin and human utrophin (1) shows that the protein sequences are 98.9% similar, with 97.1% identity. In the coding region there is 88.3% nucleotide identity between the two sequences. The amino acid conservation between mouse G-utrophin and mouse dystrophin is significantly lower.

To establish the tissue distribution of G-utrophin and determine the levels of G-utrophin RNA relative to utrophin, RNase protection assays were done on equal amounts of

A

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          10                  30                  50
CTGCTGGGGGCTGTCTGGGTGGCGTTCTTCAGTGTTTTTGTCAAATCAAGAAGTTG**ATG**G
          70                  90                  110
ACTCCCAGCCTCCTTTTCTCTAGAACCTTAAACCCCCCTTTATCTTTCAA**ATG**C**ATG**TCT
          130                 150                 170
GGAAACGAGC**ATG**CTCAGAGAACTGGCAAATCATTGTCCAGTGACCGGGAGGAAA**ATG**ATC
          190                 210                 230
GCCGCTCTTCATTTATAATGCAAATTCTGAGGACTCTTCAGAAGTGTGGTAAACTTAAAA
                M  Q  I  L  R  T  L  Q  K  C  G  K  L  K  M
          250                 270                 290
TGATGGCCGTGGTGAGAACGTCTCTGCAGAAAGTTGTGGTTTTGTTACATCGTTTACAAA
  M  A  V  V  R  T  S  L  Q  K  V  V  V  L  L  H  R  L  Q  R
          310                 330                 350
GGATGGCAGTTTCCTCTCCGCGGTACCAGAAGCTCTGCAAGGACATCCAGGCAGAAATTG
  M  A  V  S [S  P  R  Y] Q  K  L  C  K  D  I  Q  A  E  I  D
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B

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  1  MQILRTLQKC GKLKMMAVVR TSLQKVVVLL HRLQRMAVSS PRYQKLCKDI
 51  QAEIDAHNDI FKSIDGNRQK MVKALGNSEE ATMLQHRLDD MNQRWNDLKA
101  KSASIRAHLE ASAEKWNRLL ASLEELIKWL NMKDEELKKQ MPIGGDVPAL
151  QLQYDHCKVL RRELKEKEYS VLNAVDQARV FLADQPIEAP EEPRRNPQSK
201  TELTPEERAQ KIAKAMRKQS SEVREKWENL NAVTSNWQKQ VGKALEKLRD
251  LQGAMDDLDA DMKEVEAVRN GWKPVGDLLI DSLQDHIEKT LAFREEIAPI
301  NLKVKTMNDL SSQLSPLDLH PSLKMSRQLD DLNMRWKLLQ VSVDDRLKQL
351  QEAHRDFGPS SQHFLSTSVQ LPWQRSISHN KVPYYINHQT QTTCWDHPKM
401  TELFQSLADL NNVRFSAYRT AIKIRRLQKA LCLDLLELNT TNEVFKQHKL
451  NQNDQLLSVP DVINCLTTTY DGLEQLHKDL VNVPLCVDMC LNWLLNVYDT
501  GRTGKIRVQS LKIGLMSLSK GLLEEKYRCL FKEVAGPTEM CDQRQLGLLL
551  HDAIQIPRQL GEVAAFGGSN IEPSVRSCFQ QNNNKPEISV KEFIDWMHLE
601  PQSMVWLPVL HRVAAAETAK HQAKCNICKE CPIVGFRYRS LKHFNYDVCQ
651  SCFFSGRTAK GHKLHYPMVE YCIPTTSGED VRDFTKVLKN KFRSKKYFAK
701  HPRLGYLPVQ TVLEGDNLET PITLISMWPE HYDPSQSPQL FHDDTHSRIE
751  QYATRLAQME RTNGSFLTDS SSTTGSVEDE HALIQQYCQT LGGESPVSQP
801  QSPAQILKSV EREERGELER IIADLEEEQR NLQVEYEQLK EQHLRRGLPV
851  GSPPDSIVSP HHTSEDSELI AEAKLLRQHK GRLEARMQIL EDHNKQLESQ
901  LHRLRQLLEQ PDSDSRINGV SPWASPQHSA LSYSLDTDPG PQFHQAASED
951  LLAPPHDTST DLTDVMEQIN STFPSCSSNV PSRPQAM
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FIG. 2. (*A*) The 5′ sequence and conceptual translation of the G-utrophin cDNA. There are five ATG codons (boldface) in the 5′UTR preceding the open reading frame. The first 48 amino acids of G-utrophin have no apparent homology to utrophin. The four boxed amino acids form the consensus sequence for a potential phosphorylation site. Arrowhead, point at which the sequences of G-utrophin and utrophin differ. (*B*) Predicted protein sequence of G-utrophin. The amino acid sequence of G-utrophin from the first methionine predicts a 113-kDa protein.

mouse RNA by using a probe derived from G-utrophin sequence (nt 246–430) that spans the point where the two sequences diverge. With this strategy, a fully protected 185-bp fragment corresponds to the G-utrophin transcript, whereas an 88-bp fragment corresponds to the "full-length" utrophin transcript. In the brain, the G-utrophin transcript is at least five times more abundant than "full-length" utrophin (Fig. 3). G-utrophin was not detected in any other tissue except the brain, whereas the utrophin levels in the other tissues were relatively constant, except in the lung where utrophin appears more abundantly expressed.

G-Utrophin Localization by RNA *in Situ* Hybridization. To determine the sites of G-utrophin expression in the brain, oligonucleotide and RNA probes complementary to the unique 5′ sequence of G-utrophin were hybridized to sections of mouse brain. The G-utrophin transcript is readily detected in the cerebral cortex, caudate putamen, amygdala, hypothalamic area, and olfactory bulb. A strong signal is also present in the pontine, facial, vestibular and anterior olfactory nuclei, as well as in the inferior olive, whereas weaker signal is seen in the cerebellum and choroid plexus (Fig. 4 *A–C*). The same expression pattern is observed with an oligonucleotide probe complementary to the 3′-end of utrophin (common to the full-size and G-utrophin). Thus, G-utrophin appears to be the predominant form of utrophin in the adult brain, which agrees with the Northern blot and RNase protection assay results.

Emulsion autoradiography enables transcript localization at the single-cell level. This technique revealed that labeling of the cerebral cortex is predominantly in layers 3–5, and in the olfactory bulb the strongest labeling is in the granule and mitral cells. In the caudate putamen, pontine nuclei, and vestibular nuclei most neurons are labeled, whereas in the facial nucleus signal is confined to the largest neurons (Fig. 4*D*). In the cerebellum a weak signal is detected in granule cells; however, this method is not sufficiently selective to distinguish subpopulations of the positive cells in the choroid plexus.

Several criteria were used to ensure that the labeling in these studies is specific. Oligonucleotides complementary to different sequence regions (5′- and 3′-end) produced identical expression pattern in several independent experiments, and both oligonucleotide and RNA probes resulted in the same expression pattern. No signal was detected on sections hybridized with the sense cRNA probe, and incubation of sections with a 25 molar excess of the same unlabeled oligonucleotide combined with the normal concentration of labeled probe inhibited all specific binding.

The G-utrophin probe (Gu5′ E/K) was also used to establish the expression profile of this transcript during mouse embryogenesis. G-utrophin transcripts were detected at various sites, representing a subset of those detected using a 3′UTR utrophin probe that would be expected to detect all transcripts from the utrophin gene (12). G-utrophin transcripts were not detected early in development, between 8.5 days and 10.5 days post coitum. Other utrophin transcripts are apparent at these early stages and are present in the floor plate of the neural tube and in the developing sensory ganglia (12). G-utrophin transcripts are first detected from 11.5 days in the cranial nerve and

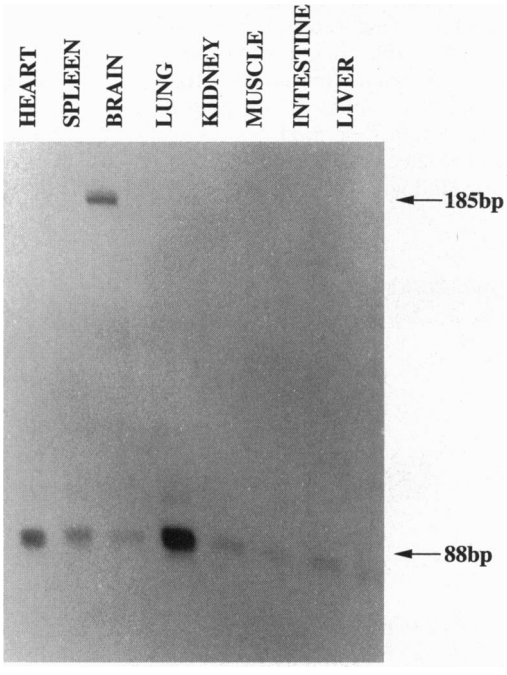

FIG. 3. Tissue distribution of the G-utrophin transcript. The RNase protection assay was used to determine the tissue distribution of G-utrophin in RNA from adult mouse tissues. The 185-bp, fully protected product, corresponding to G-utrophin, is detected only in brain RNA, whereas the 88-bp utrophin product is found in all samples but is particularly abundant in lung.

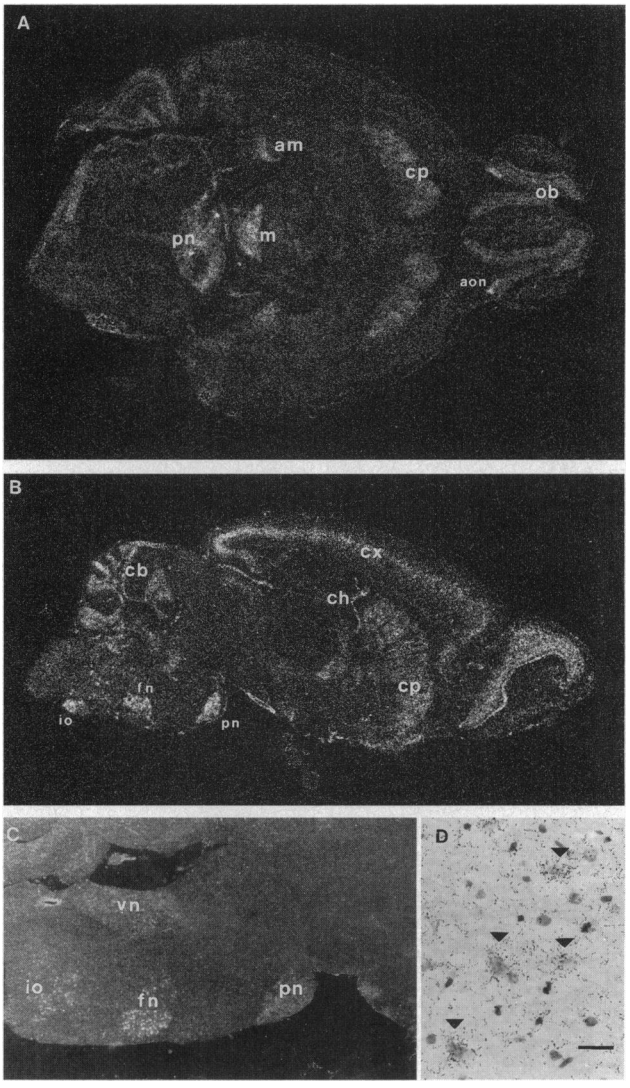

FIG. 4. Localization of the G-utrophin mRNA in adult mouse brain. Dark-field autoradiographs of horizontal (*A*) and sagittal (*B*) adult mouse brain sections. (*C*) Higher magnification of the medullo–pontine region of the brain. (×10.) (*D*) High-magnification bright-field micrograph of neurons within the facial nucleus. ob, Olfactory bulb; aon, anterior olfactory nucleus; cp, caudate putamen; m, mamillary body; am, amygdala; pn, pontine nucleus; cx, cerebral cortex; ch, choroid plexus; fn, facial nucleus; cb, cerebellum; io, inferior olive; vn, vestibular nucleus. (*D*) Arrowheads indicate G-utrophin mRNA expression in the largest neurons. (Bar = 250 μm.)

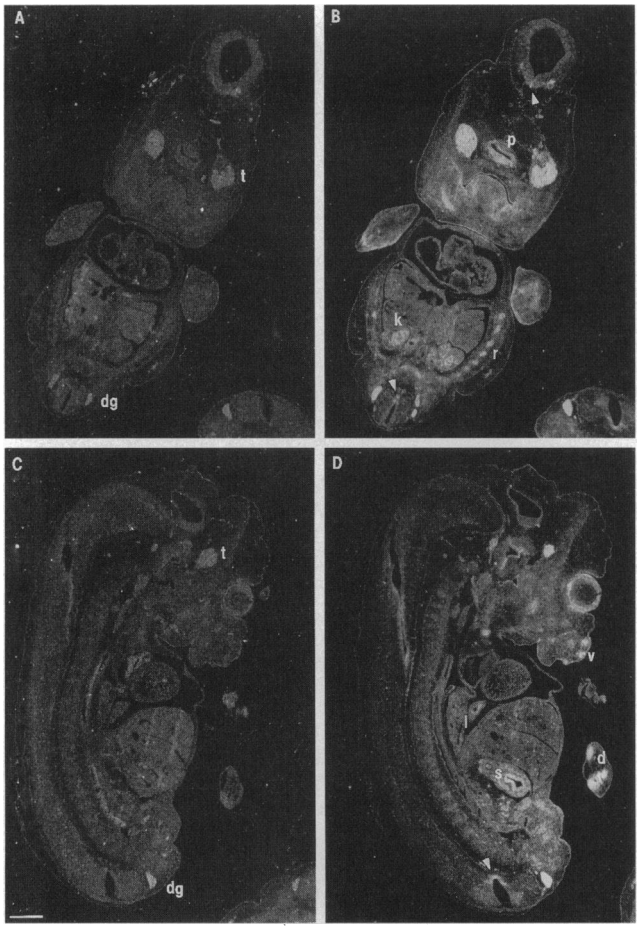

FIG. 5. Embryonic expression of G-utrophin mRNA. Frontal (*A* and *B*) and sagittal (*C* and *D*) sections through 12.5-day mouse embryos hybridized with G-utrophin specific (*A* and *C*) and utrophin 3′UTR (*B* and *D*) antisense cRNA probes. dg, Dorsal root ganglia; t, trigeminal ganglion (cranial nerve V); r, ribs; k, kidney; p, pituitary; d, digits; s, stomach; l, lung; v, vibrissae follicles. Arrowheads, expression in floor plate of neural tube. (Bar = 500 μm.)

dorsal root sensory ganglia (Fig. 5 *A* and *C*) and are confined to these sites until 15.5 days when G-utrophin mRNA also accumulates in several brain regions. This restricted pattern of G-utrophin expression contrasts with that described for utrophin in general. For example, with a 3′UTR probe, utrophin is found at a wide variety of sites by 12.5 days, including the ventral floor plate region of the neural tube, ossifying rib and facial cartilages, kidney, pituitary, stomach mucosa, digits, and vibrissae follicles (Fig. 5 *B* and *D*).

DISCUSSION

In this paper we describe the cloning of G-utrophin, a tissue-specific transcript from the utrophin locus that is expressed in specific areas of the adult brain and embryonically in sensory ganglia. G-utrophin is predicted to be a 113-kDa protein composed of the last two and a half coiled-coil repeats of the rod domain and the cysteine-rich and C-terminal domains of utrophin. G-utrophin diverges from utrophin at the same point that the short dystrophin transcript Dp116 diverges from dystrophin. This divergence is at a position that lies within intron 55 of the dystrophin sequence (18, 19). The relationship between dystrophin, utrophin, and the distally encoded products of both loci is summarized in Fig. 6. It is reasonable to conclude that G-utrophin is the autosomally encoded counterpart to Dp116. The only obvious sequence similarities between the unique N termini of these two proteins reside in four amino acids that form a consensus phosphorylation site for protein kinase C (Fig. 2*A*). This is of interest because phosphorylation of G-utrophin might modulate the assembly of cytoskeletal complexes; this modification of cytoskeletal proteins is known to disrupt protein–protein interactions (25).

G-utrophin is expressed in sensory dorsal root and cranial nerve ganglia and in defined regions of the brain—the caudate putamen, olfactory bulb, amygdala, hypothalamus, pontine nuclei, facial nuclei, and vestibular nuclei. These brain areas are involved in a diverse set of brain functions—i.e., cognition, learning, regulation of movement, coordination of autonomic and endocrine systems, and others. The sites of G-utrophin expression in the brain are clearly distinct from those where dystrophin is expressed, except for the olfactory bulb and cerebral cortex, and in this latter site G-utrophin and dystrophin are expressed in different cellular subpopulations. In addition, results presented here indicate that G-utrophin is the predominant product of the utrophin gene expressed in the adult brain. Previous immuno–histochemical studies have failed to detect utrophin or utrophin-related proteins in the areas of the brain described here. Utrophin is known to be expressed in the cerebral microvasculature (26) and is thought to be associated with the foot processes of astrocytes (10). In

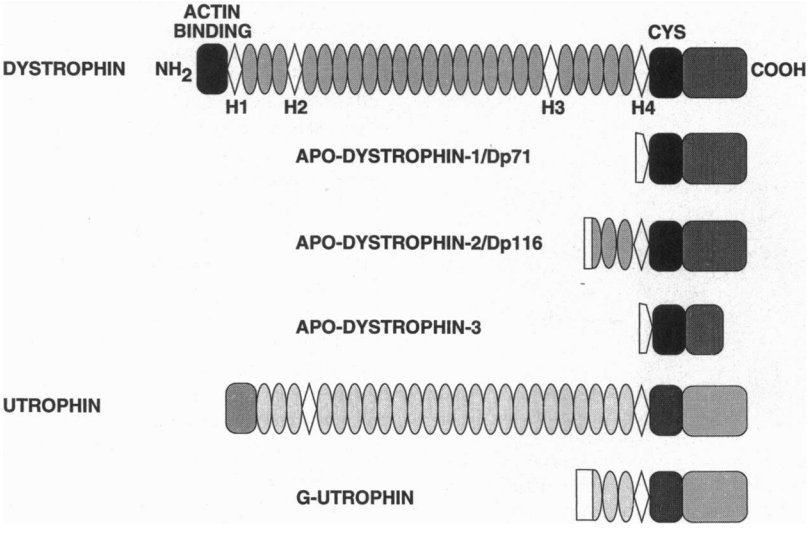

FIG. 6. Diagrammatic representation of proteins encoded by distal transcripts originating from *DMD* and utrophin (*utrn*) loci and their relationship to dystrophin and utrophin.

addition to the vascular sites of the brain, utrophin has also been detected in some neurons of the cerebral cortex (27). Within these neurons utrophin was found at the postsynaptic membrane of the dendrites and also the rough endoplasmic reticulum (27). All antibodies used in the aforementioned studies were raised against C-terminal epitopes and, therefore, should detect all utrophin isoforms, including G-utrophin. The apparent discrepancy between the mRNA expression data presented here and previous antibody-based localization studies could reflect a relatively inefficient translation of G-utrophin mRNA that might be related to the occurrence of multiple ATG codons associated with weak Kozak consensus sequences.

Since G-utrophin has the same C-terminal sequence as utrophin, it is likely that G-utrophin will also associate with the nonmuscle components of the DGC. Syntrophin (59DAP) has recently been shown to copurify with utrophin and multiple low-molecular-weight dystrophin isoforms, such as Dp71, in nonmuscle tissue homogenates (28). The 43-kDa component of the DGC, 43DAG, or β-dystroglycan, also binds to dystrophin and dystrophin-related proteins and is ubiquitously expressed. G-utrophin may be linked to the membrane by interacting with β-dystroglycan, as seems to be the case for Dp116 in peripheral nerve (29). In adult mouse brain the dystroglycan transcript colocalizes with the sites where utrophin and dystrophin are expressed (30). Surprisingly, the dystroglycan transcript was not detected in the areas where G-utrophin is expressed. This result may indicate that G-utrophin binds to an alternative functional equivalent of β-dystroglycan.

The similarities in amino acid sequence and gene structure of dystrophin and utrophin suggest that the genes have evolved from a common ancestor by gene duplication (31). Although the 900-kb utrophin gene is only one-third the size of the dystrophin gene (32), both genes are divided by many small introns that apparently occur at the same positions in the coding sequences. The discovery of G-utrophin suggests that duplication of these genes occurred after the functional appearance of the full-length 14-kb and Dp116 promoters. After duplication, divergence in the sequence and specificities of the G-utrophin and Dp116 promoters and of the extreme 5′ promoters of utrophin and dystrophin resulted in distinctive, nonoverlapping expression patterns. It now seems possible that we will find utrophin equivalents of apo-dystrophin-1 (Dp71), C-dystrophin, and P-dystrophin derived from multiple promoters at the utrophin locus.

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