Studies of Cloning, Chromosomal Mapping, and Embryonic Expression of the Mouse Rab Geranylgeranyl Transferase β Subunit

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Abstract
The mouse Rab geranylgeranyl transferase β subunit has been cloned from a mouse E8.5 embryonic cDNA library. Sequence comparison reveals 97.4% sequence identity at the amino acid level to the rat clone isolated from an adult rat brain cDNA library. This gene, given a gene symbol of Rabggtb, is mapped in the distal region of mouse chromosome 3. It is ubiquitously expressed in adult animals but displays an interesting pattern of expression during a specific time of embryonic development. The expression of this gene can be detected in the whole embryos during early embryonic stages and is specifically concentrated in the developing brain, heart, and liver between gestation stages of E11.5 and E13.5. In addition, the expression of this gene is induced by retinoic acid in a mouse embryonal carcinoma cell line, P19.

Introduction
Gene expression in early embryos of nonmammalian species has been examined extensively. In contrast, much less has been done for mammalian embryos at early stages of development due to limitation in material for experimentation and the relatively slower progress in identifying novel genes expressed at this time.

In higher animals, the study of early embryonic genes has been conducted primarily in cell cultures of embryonic origin, such as embryonal carcinoma cells (1–3) and embryonic stem cells (4, 5). However, the application of studies conducted with tissue culture systems is very limited in many respects. For instance, pattern formation and the development of a particular organ system can hardly be addressed in cell culture systems. Moreover, it is very difficult to identify genes that are important for organ development using tissue culture systems, because these genes may be expressed in a temporally and spatially specific pattern that cannot be revealed in cultures. Due to difficulty in obtaining mouse embryos at early stages of development, it has been particularly lacking in identifying genes that are involved in early gestation stages, when spatial specificity of an embryo has just begun to be determined.

In order to identify genes that are expressed in early stages of mammalian development, we have constructed cDNA libraries from mouse embryos at various gestation stages. In this study, we have constructed a cDNA library from mouse embryos at the gestation stage of E8.5. Because it is known that the most significant event during this particular stage of development is the specification of each body segment and the beginning of organ formation, we have screened this library with probes prepared from a Drosophila cDNA of a segmentation gene, paird (6). Interestingly, we have cloned the β subunit of the mouse Rab geranylgeranyl transferase (also known as geranylgeranyl transferase type II) from this library. The expression of this gene can be detected in the whole embryos before E11.5 and is particularly enriched in the brain, the heart, and the liver of embryos between gestation stages E11.5 and E13.5. In addition, this gene appears to be induced by retinoic acid in a mouse embryonal carcinoma cell line P19.

Results
Construction and Screening of an E8.5 Mouse Embryonic cDNA Library. A total of 450 mouse embryos at the stage of E8.5 were collected, and poly(A) RNA was prepared for the construction of a cDNA library in Agt10 vectors at the EcoRI site. This library yielded a total of 1.7 × 10^6 independent clones. To screen the library, 5 × 10^5 clones were plated and hybridized with 32P-labeled probes prepared from Drosophila paird cDNA, under a low-stringent hybridization condition (43% formamide at 37 C). Subsequently, five clones were purified and sequenced. Detailed sequence analysis of these clones shows that all of these cDNAs are derived from the same species of transcripts but terminate at various 5' ends. The largest clone contains an insert of approximately 1.1 kb in length and has an open reading frame spanning 304 amino acid residues. Sequence analysis of this cDNA, compared to the Drosophila paird cDNA, reveals no significant homology at either nucleotide or amino acid level, except in two regions where identical sequences are found in 16 bp of 20 and 18 bp, respectively (underlined and labeled with hom-1 and hom-2 in Fig. 1). However, after searching through GenBank, it was found that this cDNA contains an open reading frame with 97.4% amino acid identity (296 of 304) when compared to the rat Rab geranylgeranyl transferase β subunit sequence (Ref. 7;

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3 The abbreviations used are: kb, kilobase(s); bp, base pair(s); RT-PCR, reverse transcription-PCR; RA, retinoic acid.
a

TGCCTCTATG GCCAAGGAA AGATGTTAT GAATATCAGA TGCTCTGATA CCTGAAATG AGCGGCCGCT

b

ATTGCGGCT CAGCTTTGTT GACGCTATGG GACAGCTCAG TCCGATGACC GAAAGAAATA TCCGTTGATT

Y W G L T V M D L M G Q L H R M N R E E I L V F 74

hom-1

TATCAAGTGG TGCCAGAATG AGACGCTTGC TGGAGAAGA ACAACTGAT GCCAGAAGAG 97

ACGGCTAC GCTGCAAGAT CTTTCAGCT TCTGCAAG TACGCAGTAG AAGGCCTGG

T L S A V Q I L T L Y D S V H V I N V D K V V 120

c

CTTATGTTCA GAGGCTCAG AAAGAAATG GCCCTTGGTG TGGAGCAAT TGGGAGAATA TTGATAACAG

A Y V Q S L K E D G S F A G D I W G E I D T R 144

ATTCTCATT TGGCGGCTG GAACCTGAC TCTTTCGGTC AAGCTTGGAT CATTAAATGT GAAAAGGCGC

F S F C A V A T L A L G K D A I N V E K A 167

ATTGATTGTG TTTTGCTCTG CATGAACTT TGAGTGGAT TGGGCGCAGT ACAGGCTCT GCCAATCAG

I E F V L S C M F D G G F G C R P G S E H 190

CTGGGCAAT CATATTGTTG AAAGAATTG TGCCTTGGTC TGGAGCAAT TGGGAGAATA TTGATAACAG

A G Q I Y C T G F L A I T S Q L H Q N S D L 214

ACTGTTGGT TGGCTTTTGT GGCGGCGTT GCCCTTGGTC GCACTCAATG GAAGACGCGA GAAGTACCA

L G W W L C E A Q L P S G A L N G R E K L P 237

GATGCTTGTG ATTCGATTG TGATTTTGGCC TTCCTAAAGA TCACTGGGAG ACTTCATTGG ATTCGATCGC

D V C Y S W W V L A S L K I I G R L H W I V S 260

AAAACGCTT AAGATTCTAC TTTGCCATGC AAATGAGAA GAGCCGAGA TTGTCAAGAA GACCAGAGAG

E K L R S F I L A C Q D E E T G G F A D R P G D 284

TATGTTGAT CCGTTTCTA CTTATGGATG CATTGCTGGT TGGTACCTT GCGAGAAATG CAGATCAAG

M V D P F H T L F G I A G L S L L G E E Q I K 307

CCTGTTGCC CTGTCTCTTG TATGCAGAA GAGGTTCTCC GAGGGGGAAG TCCGAGCCT TGGCATATG

P V S P V F C M P E E V L Q R V N V Q P E L V 330

GCTAGTCA AAGGGAGAG ATGTGATTGT GCAATATGAA TCTACGCTGT ATTTGAAGTG TTTTCAAGAC

S T E R 396

CTAGAGTGA CTAAAAATTT TGATATCAT ATGTTAATGA TAAATTATAT AAGATTTGTA ATATAAAATAT

GTTGTATTTA GAGAAGAATC

Fig. 1. The nucleotide sequence of the mouse Rab-geranylgeranyl transferase β subunit and the comparison of the amino acid sequence to the rat protein. The mouse cDNA begins at amino acid number 28, according to the rat clone (Ref. 7). The mouse amino acid sequence (m) is shown under the DNA sequence, and the rat sequence (r) is shown under the mouse sequence. The label "r-" in the rat sequence indicates identical amino acid residue in each position. The primers used in RT-PCR are underlined and labeled as "a" and "b." The oligonucleotide used in in situ hybridization is underlined and labeled as "hom-1" and "hom-2," respectively. A poly(A) signal is shown as bold letters and underlined. This sequence has been submitted to GenBank under the accession number X12922.

amino acid number 28 to 331, according to the rat sequence. This mouse cDNA (labeled with m) begins at amino acid position 28 according to the rat sequence (labeled with r). A gene symbol for this mouse cDNA has been proposed to The Jackson Laboratory and accepted as Rabggtb in order to conduct chromosome mapping. The sequences of oligonucleotides for PCR and in situ hybridization are underlined and labeled with a, b, and c. A poly(A) site is labeled with bold letters and underlined.

Genomic Southern Blot Analysis. In order to gain an insight into the genomic structure of this gene, a genomic Southern blot was conducted using probes prepared from this cDNA. As shown in Fig. 2, a strong hybridizing signal is seen on a single BamHl band (Lane 2), a single SacI band (Lane 3) and two EcoRI bands (Lane 4). This blot has been washed in 0.1X SSC at 65°C. This washing condition has been used in the following experiments involving blot hybridization, such as Northern blot and RT-PCR analysis.

Mouse Chromosome Mapping. The mouse chromosomal location of the Rab geranylgeranyl transferase β subunit (Rabggtb) was determined by interspecific backcross analysis using progeny derived from matings of (C57BL/6) × 129/SvJ.
Fig. 2. Genomic Southern blot analyses. Mouse genomic DNA (5 μg each) has been digested with restriction enzymes BamHI (Lane 2), Sac I (Lane 3), and EcoRI (Lane 4), followed by Southern transfer and hybridization with the mouse cDNA Rabggtb probe. The 4.4-kb M. spretus Bgl RFLP (see “Materials and Methods”) was used to follow the segregation of the locus in backcross mice. The mapping results indicated that Rabggtb is located in the distal region of mouse chromosome 3 linked to Egf, Nfkb1, and Rpe65. Although 128 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 3), up to 182 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere-Egf/9/182-Nfkb1-15/159-Rabggtb-4/137-Rpe65. The recombination frequencies (expressed as genetic distances in centimorgans ± SE) are: -Egf-5.0 ± 1.6-Nfkb1-9.4 ± 1.4-Rpe65.

Northern blot analysis was conducted as shown in Fig. 4. The blot was hybridized, first with probes prepared from mouse Rabggtb cDNA (Fig. 4A) and then with actin-specific probes (Fig. 4B). Fig. 4C shows the photograph of the agarose gel used for this blot. The arrows in Fig. 4A indicate the positions of 28S and 18S rRNA, and the arrow in Fig. 4B indicates the position of the 18S rRNA. The Rabggtb gene is expressed in all the major organs that have been examined, including stomach (Fig. 4, Lane 1), testis (Fig. 4, Lane 2), kidney (Fig. 4, Lane 3), lung (Fig. 4, Lane 4), liver (Fig. 4, Lane 5), heart (Fig. 4, Lane 6), thymus (Fig. 4, Lane 7) and brain (Fig. 4, Lane 8), with the highest level of expression in

Fig. 3. Rabggtb maps in the distal region of mouse chromosome 3. Rabggtb was placed on mouse chromosome 3 by interspecific backcross analysis. The segregation patterns of Rabggtb and flanking genes in 128 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 128 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the [(C57BL/6J × M. spretus) × C57BL/6J] parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of a M. spretus allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 3 linkage map showing the location of Rabggtb in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci cited in this study and can be obtained from Genome Data Base, a computerized database of human linkage information maintained by The William H. Welch Library of The Johns Hopkins University (Baltimore, MD).
the heart and the brain of adult animals. The size of its transcript is determined to be approximately 1.8 kb.

**Rabggtb Gene Expression in Embryos.** Because this cDNA was isolated from an E8.5 cDNA library, it would be interesting to examine the expression of this gene during developmental stages. In order to detect small amounts of RNA isolated from early embryos, the most sensitive detection method, RT-PCR, was used as described in “Materials and Methods.” As shown in the upper panel of Fig. 5, the expression of Rabggtb, as demonstrated by the amplification of DNA fragments in the size of 150 bp (Fig. 5, upper panel, arrow) is readily detectable in E7.5 embryos (Fig. 5, Lane 2), the earliest time when embryos can be dissected for the isolation of RNA, as well as all the later stage embryos (Fig. 5, Lanes 3–9). The expression level is significantly elevated beginning at E11.5 (Fig. 5, Lane 4), levels off at E14.5 (Lane 7), and decreases to a basal level in E19.5 embryos (Lane 9). Fig. 5, Lane 10 shows a control PCR reaction containing all the components except RNA. The actin messages have been amplified with specific primers at the correct size of 270 bp in each PCR at a relatively similar level (the upper band in the lower panel which shows a picture of ethidium bromide-stained agarose gel used for this Southern blot). Fig. 5, Lane 1 is an isotope-labeled DNA size marker derived from HindIII-digested λ DNA, and Fig. 5, Lane 11 shows a low molecular weight DNA size ladder. In order to compare the expression level between embryos and adult organs, a Northern blot was conducted as shown in Fig. 6. Fig. 6A shows the results using Rabggtb cDNA specific probes, Fig. 6B shows the results using actin specific probes, and Fig. 6C shows a photograph of the agarose gel used for this blot. Fig. 6, arrows on the right indicate the position of rRNA, and arrow on the left indicates the position of Rabggtb-specific transcripts. It is obvious that the expression in postnatal brain, either at 4 weeks (Fig. 6, Lane 1) or 6 weeks (Fig. 6, Lane 2), is much lower than that in E11.5, E12.5, and E13.5 whole embryos (Fig. 6, Lanes 3, 5, and 4, respectively). An E13.5 embryonic liver sample is shown in Fig. 6, Lane 6.

**Spatial Localization of Expression in Embryos by in Situ Hybridization.** In situ hybridization was conducted to examine the spatial pattern of expression of the mouse Rabggtb gene in mouse embryos. An anti-sense oligonucleotide
sections, the expression is detected in the whole embryos including extraembryonic membranes at E9.5 and E10.5. By E11.5, the expression is elevated in developing brain (Fig. 7C, a, b, and c), heart (Fig. 7C, d) and liver (Fig. 7C, e). The expression patterns at E12.5 and E13.5 are very similar (data not shown). The same pattern of expression is observed with another antisense probe specific to the COOH terminus (data not shown). Therefore, it is concluded that the mouse Rabggtb gene is expressed in the whole embryos before E11.5, and its expression is highly elevated in developing brain, heart, and liver between E11.5 and E13.5. The expression of this gene then levels off evenly in the whole embryos by E14.5.

Effect of RA on Mouse Rabggtb Gene Expression in P19 Cells. Having observed interesting changes in the expression pattern of Rabggtb gene in the brain, heart, and liver within a particular time window of embryonic development (between E11.5 and E13.5; Figs. 5, 6, and 7), we thought that it would be interesting to examine if the expression of this gene was related to any cellular events that might be relevant to embryonic development and to explore factors that might regulate its level of expression. RA is a natural morphogen and is able to induce P19 mouse embryonal carcinoma cell differentiation into various cell types including, most notably, neurons and beating heart muscle cells (2, 3). In addition, null mutation at retinoic acid receptor RXRa gene has been shown to affect heart morphogenesis in gene-targeted animals (9). It was speculated that expression of Rabggtb gene could be affected by RA in P19 system. As a first step, we examined whether the mouse Rabggtb gene responded to RA treatment in this cell differentiation model. P19 cells were plated at a density of 10⁶ cells/10-cm dish as described by Wei et al. (3), and 10⁻⁷ m RA was added 12 h later. At various time points, cellular RNA was isolated, and 50 μg from each sample were analyzed by Northern blot hybridization as shown in Fig. 8. RNA samples isolated at 0, 12, and 24 h and days 2, 3, 4, and 6 are shown in Fig. 8, Lanes 1–7, respectively. Fig. 8A shows the results using Rabggtb-specific probes, Fig. 8B shows results using actin-specific probes, and Fig. 8C shows the photograph of the agarose gel used for this blot. It is obvious that this gene is induced by RA for approximately 5–10 h within 12 h of RA treatment, and the induced level of Rabggtb expression begins to level off at the 4th day of treatment. Actin expression remains relatively constant within 4 days of RA treatment. Cells treated with RA for a longer period of time begin to die as actin expression begins to decrease at day 6 (Fig. 8, Lane 7).

Discussion

This study reports the isolation of a mouse embryonic cDNA encoding 304 of 331 amino acids of the β subunit of mouse Rab geranylgeranyl transferase, which has been given a gene symbol of Rabggtb. Sequence comparison (Fig. 1) shows 97.4% identity at the amino acid level to a reported rat Rab geranylgeranyl transferase subunit β for the 304 amino acid sequence (7). Although this cDNA has been isolated using probes prepared from Drosophila paired cDNA, this cDNA appears to have no significant homology to the Drosophila gene. It has been later determined that the Drosophila cDNA is able to cross-hybridize with this mouse cDNA under a low-stringency hybridization condition used during library screening (37°C; 43% formamide), but the hybridizing signals can be washed off under a higher strin-
gent condition (65°C; 0.1X SSC). Detailed sequence analysis to search for homologous sequences between the mouse cDNA and the Drosophila cDNA has revealed two short homologous regions, each with nucleotide sequence identity of 16/18 and 16/20, respectively. It is possible that cross-hybridization is due to these short homologous sequences.

Genomic Southern blot analysis conducted under a high-stringency condition (Fig. 2) has shown a single hybridizing band in DNA digested with BamHII or with SalI, which would suggest that this cDNA does not have other homologous sequences in the mouse genome that can be detected under this high-stringency washing condition. Thus, this condition has been used in all of the subsequent experiments using blot hybridization techniques in order to ensure the specificity of hybridizing signals. However, the possibility of the presence of some remotely related sequence cannot be ruled out because this cDNA encodes only two-thirds of the entire transcript, based upon the size (approximately 1.8 kb) of the transcript detected by Northern blot analysis.

The size of the mouse transcript, determined to be approximately 1.8 kb in this study, agrees with the size of the reported adult rat messages (7). This mouse gene is ubiquitously expressed in adult tissues as demonstrated by Northern blot analysis but with higher levels of expression observed in the heart and brain (Fig. 3). Interestingly, the expression of this gene begins very early during embryonic development, significantly increases in the developing brain, heart, and liver between E11.5 and E13.5, and levels off at E14.5 (Figs. 5 and 7). Compared to its expression in adult tissues, embryonic expression between E11.5 and E13.5 is at least 2-fold higher (Fig. 6).

The mouse Rabggtb has been mapped in the distal region of mouse chromosome 3 linked to Egl, Ntkb1, and Rpe65. We have compared our interspecific map of chromosome 3 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations. Rabggtb mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (data not shown). The distal region of mouse chromosome 3 shares a region of homology with human chromosome 4q and 1p (summarized in Fig. 3). In particular, Ntkb1 has been placed on human 4q24 and Rpe65 on 1p31. The mapping of Rabggtb between Ntkb1 and Rpe65 in the mouse suggests that the human homologue of Rabggtb will reside on one of these two human chromosomes.

The Rab geranylgeranyl transferase attaches a 20-carbon isoprenoid moiety to COOH-terminal cysteins in small GTP-binding proteins that terminate in Cys-Cys or Cys-X-Cys (7). These target proteins include Rab1A, which resides in the endoplasmic reticulum and Golgi complex, and

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4 Compiled by M. T. Davieson, T. H. Roderick, A. L. Hillyard, and D. P. Doolittle and provided from GBASE, a computerized database maintained at The Jackson Laboratory (Bar Harbor, ME).
Rab3A, which is a component of synaptic vesicles (10). Geranylgeranylation is required for these target proteins to adhere to the cytoplasmic leaflets of membranes. The functional enzyme is made up of component A and component B, and component B contains subunits α and β. However, the expression of α and β subunits is not always at an equal molar ratio (7). The strong expression of the β subunit of this enzyme in the developing heart, brain, and liver between the stages of E11.5 and E13.5 is interesting. The liver at the stage of E12 is seen to be a well-differentiated organ, both structurally and functionally. More of its structure at this time of development is occupied by functional hepatic parenchymal tissue than previous stages. The brain region is now expanding and differentiating, and cellular migration from the mantle zone into the overlying marginal zone begins to be seen. At this time, the overall size and complexity of the heart begins to increase, and heart beats can be detected. In addition, RA is believed to be involved in the development of several organ primordia, including the brain and heart (9). Using an embryonal carcinoma cell differentiation system, P19, it is observed that the expression of this mouse gene is induced by RA fairly early (within 12 h). Strong expression of this gene in developing brain and heart, and its induction by RA in P19 cells, would make it interesting to study the relationship between the RA signaling pathway and protein geranylation during developmental stages in the future. Although the β subunit is strongly expressed in these developing organs, which may suggest a role for geranylgeranylation in the development/maturation of these organs, it remains to be determined if and how the other subunit, α, is expressed at this time.

Materials and Methods

Materials. Embryos were obtained from CF1 female mice at the gestation date of E8.5 for the construction of a cDNA library. Cryosections were obtained from CD1 embryos at various developmental stages for in situ hybridization. Radiosotopes and a random priming kit were purchased from NEN. P19 cells were maintained in α-MEM supplemented with 2.5% FCS and 7.5% calf serum as described previously (3). RA was purchased from Sigma Chemical Co. and prepared in ethanol as a 10⁻⁴ M stock solution.

cDNA Libraries. Poly(A) RNA was prepared from E8.5 embryos, and a cDNA library was constructed in Agt10 vectors at the EcoRI site using a standard protocol (11).

Cloning and Sequence Analyses. The cDNA library was screened under a low-stringent hybridization condition (at 37°C and with 37% formamide). The probe was prepared by labeling the Drosophila paird cDNA fragment (6) using a random primer labeling kit. Positive clones were purified, and the inserts were released by EcoRI digestion. The inserts were subcloned into a pGEM plasmid vector for DNA sequencing. DNA sequencing was conducted by the dyeoxy chain termination method (12), and DNA sequences were analyzed using a sequencing software such as DNASTAR.

Southern Blot and Northern Blot Analyses. Genomic DNA was isolated from mouse liver and analyzed by standard Southern blot hybridization (13) under a high-stringency hybridization condition (at 50°C and with 50% formamide). RNA was isolated from mouse tissues and P19 cells by the method of Charron as described by Wei and Lee (14). Embryonic RNA was isolated and purified with an RNAzol solution (Tel-Test, Inc., Friendswood, TX) as described (15). Southern blot, Northern blot, and slot blot analyses were conducted under the same high-stringency condition, and all of the blots were washed in 0.1X SSC at 65°C.

Interspecific Mouse Backcross Mapping. Interspecific backcross progeny were generated by mating (C57BL/6J × M. spreitus) F₁ females and C57BL/6J males as described (8). A total of 205 N₂ mice were used to map the Rabggtb locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, and hybridization were performed essentially as described (16). All blots were prepared with Hybond-N⁺ nylon membrane (Amersham). The probe, an ∼650-bp EcoRI fragment of mouse cDNA, was labeled with [α³²P]dCTP using a nick translation labeling kit (Boehringer Mannheim); washing was done to a final stringency of 1.0X SSC (15.4 mM Na₂HPO₄, 4.6 mM NaH₂PO₄, 17.1 mM sodium citrate, and 120 mM NaCl)-0.1% SDS at 65°C. A major fragment of 2.9 kb was detected in BglI-digested C57BL/6J DNA, and a major fragment of 4.4 kb was detected in BglI-digested M. spreitus DNA. The presence or absence of the 4.4-kb M. spreitus-specific BglI fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to Rabggtb including epidermal growth factor (Egf), nuclear factor of kappa light chain gene enhancer in B cells 1 (Nkib1), and retinal pigment epithelium (M, 65,000; Rpe65) has been reported previously (17). Recombination
distances were calculated as described (18) using the computer program PRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

**RT-PCR.** An established RT-PCR procedure was used to analyze the expression of this gene in small amounts of mouse embryonic RNA as described previously (15). One μg of RNA was reverse transcribed using an oligo (dT) primer in 50 μl reaction mixture, and 2 μl of the reaction product was used for subsequent PCR in 100 μl reaction mixture. The 5' primer was designated as "a" (5'GAATTC-CGCTTCTATGGCTC3') and the 3' primer was designated as "b" (5'GGATTTCTTCTCGGTTCATG3'). These primers flank a fragment of 150 bp in the cDNA and span an intron in the genomic DNA. Therefore, a fragment of 150 bp could only be amplified from reverse transcribed cDNA. Cycles were optimized as: 94°C, 3 min; 55°C, 1 min; 72°C, 1 min for 1 cycle, and followed by 31 cycles of 94°C, 1 min; 55°C, 1 min; and 72°C, 1 min. Following PCR, 20 μl of each reaction was loaded on a 2% agarose gel, followed by Southern blot hybridization with Rabggtb-specific probes. An actin primer pair was included in each reaction as an internal control for the quality and quantity of RNA samples as described previously (15).

**In Situ Hybridization.** The spatial pattern of expression was examined by in situ hybridization on cryosections of mouse embryos, using a protocol modified from Wilkinson (19). Embryos were freshly frozen in OCT and immediately stored at −20°C until the time of sectioning. Serial sections of 10 μm were obtained from embryos at various developmental stages using a LEICA cryostat. An antisense oligo (5'GGCTCTGAACATAGGCCAACGCTTTATCTAGCTGA-3') designated as "c" was labeled with [35S]dATP by terminal deoxynucleotidyl transferase (Boehringer Mannheim), followed by purification from an NENSOBR 20 column. Probes were added at the specificity of approximately 1 X 107 cpm/ml. Hybridization was carried out at 42°C in the presence of 50% formamide for 12 h. Washing was conducted in 1X SSC at 55°C. Following hybridization, slides were dipped in NTB2 emulsion and developed with Kodak Developer D-19. Slides were then stained with bisbenzimide to observe the morphology (20). Slides were examined with an Olympus microscope under a darkfield illumination system supplemented with a fluorescent light source.

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