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Genomic Structures and Characterization of Rae1 Family Members Encoding GPI-Anchored Cell Surface Proteins and Expressed Predominantly in Embryonic Mouse Brain[1]

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Rae1α, Rae1β, and Rae1γ cDNAs isolated from retinoic acid-treated mouse embryonal carcinoma F9 cells encode cell surface proteins sharing partial homology with MHC class I molecules, and mRNAs corresponding to these cDNAs were detected exclusively in early mouse embryos, especially in the head region. To initiate studies on their roles, the *rae1α* gene and the genomic DNAs covering the complete coding regions of the *rae1β* and *rae1γ* genes were isolated and their structures were analyzed. Although the coding regions of the three *rae1* genes were highly homologous, the restriction map of the 5′-end region of the *rae1α* gene differed from that of the *rae1β* and *rae1γ* genes. The *rae1* family members were mapped by FISH on mouse chromosome 10A4 region. Genomic DNAs hybridizable with a Rae1 cDNA were not detected in rat and human. *Rae1* genes were preferentially expressed in early mouse embryos, preferentially in the brain, and RAE1 proteins were anchored on the cell surface by a glycosyl phosphatidylinositol (GPI)-tail, a feature shared by important cell surface ligands.

Key words: cell surface protein, early mouse embryo, gene family, GPI-anchor, Rae1.

The retinoic acid (RA)-induced differentiation of embryonal carcinoma (EC) cells provides an appropriate model system for studying early mammalian development (*1-3*). Numerous genes related to mammalian development have been identified from RA-induced EC cells (*4-7*), and further analyses have assigned specific roles to some of these (*8-10*).

Recently, we reported the isolation and characterization of highly homologous Rae1α, Rae1β, and Rae1γ cDNAs from RA-induced F9 cells (*4, 5*). The *rae1* genes are expressed exclusively in early mouse embryos, and the RAE1 proteins are cell surface proteins (*5*). The deduced RAE1 proteins share a partial homology with MHC class I molecules and their extracellular domains contain five *N*-linked and three *O*-linked potential glycosylation sites, and five cysteine residues (*5*). These features suggest that RAE1 proteins are cell surface glycoproteins, probably involved in the RA-regulated systems, and with roles in early mouse embryogenesis.

To initiate studies on the roles of the Rae1 family members in early embryogenesis, genomic DNAs covering the *rae1* genes were isolated and their structures were analyzed. We found that expressions of the Rae1 mRNAs are predominant in brains of embryos 9 and 10 days post-coitum (d.p.c.), and that RAE1 proteins are glycosyl phosphatidylinositol (GPI)-anchored cell surface glycoproteins. Based on our findings, we speculate that RAE1 proteins function as cellular communication molecules during early embryogenesis of the mouse.

MATERIALS AND METHODS

Cell Culture and DNA Preparation—Details on the culture of mouse embryonal carcinoma F9 cells have been reported (*4*). High molecular weight DNAs were extracted from human peripheral polymorphocytes, rat liver, undifferentiated F9 cells, chicken embryos, *Xenopus* liver, *Drosophila* Schneider cells, yeast, and *Escherichia coli*, following procedures described in Ref. *11*.

Isolation of Genomic Clones—A λFIXII/mouse genomic DNA library constructed from partial *Sau*3AI digest of the 129/SVJ mouse liver DNA (1×10^9 pfu/ml) was purchased from Stratagene and screened under stringent conditions: hybridization was carried out in 6×SSC/5×Denhardt's

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Abbreviations: aa, amino acid; d.p.c., days post-coitum; EC, embryonal carcinoma; FISH, fluorescence *in situ* hybridization; GPI, glycosyl phosphatidylinositol; GST, glutathione *S*-transferase; kb, kilobase; kd, kilodalton; MHC, major histocompatibility complex; nt, nucleotide(s); PCR, polymerase chain reaction; PI-PLC, phosphatidylinositol-specific phospholipase C; RA, retinoic acid; Rae1, retinoic acid early inducible cDNA clone 1; *rae1*, the gene corresponding to Rae1 cDNA; STP-rich, serine-threonine-proline-rich; *tsp*, transcription start point(s).

solution/0.1% SDS at 65°C and filters were washed with an excess of 0.1×SSC/0.1% SDS at 65°C. The Rae109 cDNA (*5*) used as a probe was labeled with ^{32}P by random priming. Genomic DNAs were extracted from purified phage clones and used for restriction enzyme mapping. The fragments containing exons were identified by Southern blot hybridization, and subcloned into pBluescript II KS (Stratagene) vector for sequencing.

Southern Blot and Zoo Blot Analyses—For Southern blot analysis of *rae1* genes, mouse genomic DNAs were digested to completion with restriction enzymes (*Eco*RI, *Bam*HI, or *Hin*dIII), electrophoresed on a 0.7% agarose gel, and transferred to nylon membranes. ^{32}P-labeled Rae109 cDNA (*5*) or DNA fragments prepared from cloned DNAs (Fig. 1A, probes 1-4) were used as probes. Hybridization was performed under stringent conditions as described above.

For zoo blot analysis, high molecular weight DNAs were digested to completion with *Eco*RI, separated on 0.8% agarose gels and transferred to nylon membranes (Amersham). The Rae109 cDNA and a 1.5-kb *Xba*I fragment covering exons 1 to 3 of the B6 mouse H-2K^b gene (*12*) were used as probes. Hybridization was performed under low stringent conditions: 6×SSC/5×Denhardt's solution/0.1% SDS at 55°C, and subsequent washing was done with 2×SSC/0.1% SDS at 55°C.

DNA Sequencing—DNA inserts subcloned in the pBluescript II KS vector were sequenced using T7 Sequencing Kits (Pharmacia) with the T7-, T3-, SP6-, and M13 reverse-primers.

Primer Extension Analysis—A 25-mer oligonucleotide corresponding to the antisense sequence of the *rae1α* gene from nucleotide (nt) numbers +91 to +115, which was named pe-1 (Fig. 2B, 5′-TCCTGCGAAGCTGGAGTGTCT-GTCT-3′), was used for primer extension analysis. Poly-(A)$^+$ RNAs were prepared from F9 cells treated with RA for 72 h and were analyzed as described by Triezenberg (*13*).

Chromosomal Mapping—The direct R-banding fluorescence *in situ* hybridization (FISH) method was used for chromosomal assignment of the *rae1* gene family as described (*14, 15*). A 3.0-kb genomic DNA fragment prepared from the *rae1β* gene (Fig. 1A, probe EIX2) and covering the highly conserved region from exons 6 to 9 was labeled with biotin 16-dUTP by nick translation (Boehringer Mannheim) following the manufacturer's protocol, and was used as a probe.

Preparation of Digoxigenin-Labeled RNA Probes and Whole-Mount In Situ Hybridization (16)—Digoxigenin-labeled RNA probes were prepared as described in manuals provided with Boehringer Mannheim's digoxigenin-labeling kit (Boehringer Mannheim). Rae1 cDNA from nt 292 to 819 (*5*), which corresponds to nt +118 to +845 of the *rae1α* genomic DNA (Fig. 2B), was subcloned into the *Pst*I-*Eco*RI sites of pSPT19. After linearizing the plasmid DNA with *Hin*dIII, the digoxigenin-labeled antisense probe was synthesized using SP6 RNA polymerase. The same Rae1 cDNA fragment was subcloned into the *Pst*I-*Eco*RI sites of pSPT18 and, after linearizing the plasmid DNA with *Eco*RI, the sense probe was similarly synthesized. Amounts of the synthesized RNAs were quantitated using an anti-digoxigenin antibody after spotting aliquots of the RNAs and the digoxigenin-labeled RNA standard onto a nylon membrane (Amersham). Procedures for the whole-mount *in situ* hybridization of mouse embryos are described in Ref. *16*.

Immunocytochemical Examination and Phosphatidylinositol-Specific Phospholipase C (PI-PLC) Treatment—RAE1 proteins on the cell surface were detected essentially as described earlier (*5*). To obtain evidence for GPI linkages between RAE1 proteins and cell membranes, cells were pretreated in complete tissue culture medium containing DNase I (200 μg/ml, Takara) and 2-mercaptoethanol (5×10^{-5} M) for 60 min at 37°C with 200 mU/ml of PI-PLC (Toagosei) (*17*).

Preparation of Anti-RAE1 Antibody—pGEX-3X, a prokaryotic GST gene fusion vector, purchased from Pharmacia, was digested with *Sma*I, and ligated to the *Bam*HI-*Eco*RI fragment of Rae1 cDNA (*5*) after blunt-ending it with the Klenow fragment of *E. coli* DNA polymerase I. This fragment encodes RAE1α protein from aa number 43 to 142 and the recombinant encodes a 38 kDa GST-RAE1 fusion protein. This fusion protein was solubilized with 1.5% sarkosyl and purified using Glutathione Sepharose 4B. One milligram of the GST-RAE1 fusion protein was used to immunize a rabbit.

Immunoblot Analysis—F9 cells stably transfected with Rae1 cDNA were cultured in 35-mm dishes, harvested, dissolved in 400 μl of Laemmli buffer and boiled for 2 min. Proteins were electrophoresed through 13% polyacrylamide gels, then transferred onto a membrane (Immobilon) by applying a constant voltage of 20 V for 16 h. The membrane was processed for antigen detection by chemiluminescence, using Renaissance (Dupont NEN). RAE1 antisera and anti-rabbit horseradish peroxidase-conjugated secondary antibody were used to detect RAE1 proteins.

To examine the glycosylation of RAE1 proteins, F9 cells stably transfected with Rae1 cDNA were cultured as described above, and after adding tunicamycin, an inhibitor of glycosylation, to the medium at the concentration of 0.5, 1.0, or 2.0 μg/ml, the cells were cultured for 1 more day and treated as described above.

RESULTS

Genomic Structures of rae1 Family Members—Phage clones carrying *rae1* genes were isolated by screening a gene library constructed on 129/SVJ mouse liver DNAs, using as a probe Rae109 cDNA (*5*). Restriction endonuclease mapping with *Eco*RI, *Bam*HI, and *Hin*dIII was done for each positive clone, and the exon-containing regions were identified by Southern blot hybridization. Exon-intron organization was determined by sequencing (Fig. 1A). From the restriction maps and sequence data, three highly homologous, but different *rae1* genes were identified, and the nt sequences of all the exons were in good agreement with those of the three cDNAs (*5*) (data not shown).

The *rae1α* gene is split into 9 exons, spanning approximately 25 kb (Fig. 1A). The open reading frame is located within exons 5, 6, 7, and 8. The leader sequence is encoded by a part of exon 5, the extracellular region is encoded mainly by exons 6 and 7 (exon 6 encodes the α1 domain and exon 7, the α2 domain), and a part of exon 8 encodes the STP-rich region and transmembrane domain (*5*) (Fig. 1A).

The cloned genomic DNAs covering the *rae1β* and *rae1γ*

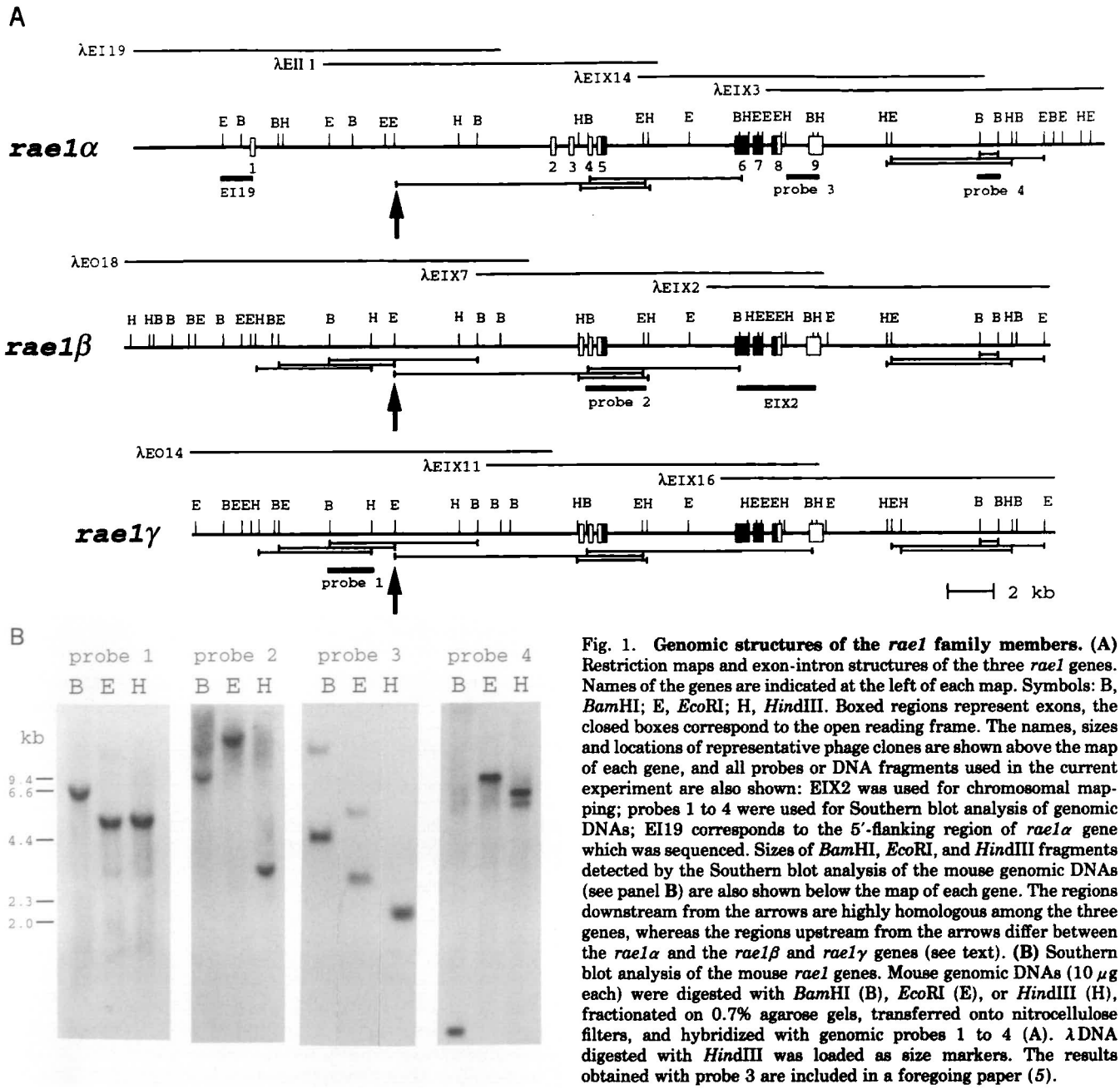

Fig. 1. **Genomic structures of the *rae1* family members. (A)** Restriction maps and exon-intron structures of the three *rae1* genes. Names of the genes are indicated at the left of each map. Symbols: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII. Boxed regions represent exons, the closed boxes correspond to the open reading frame. The names, sizes and locations of representative phage clones are shown above the map of each gene, and all probes or DNA fragments used in the current experiment are also shown: EIX2 was used for chromosomal mapping; probes 1 to 4 were used for Southern blot analysis of genomic DNAs; EI19 corresponds to the 5′-flanking region of *rae1α* gene which was sequenced. Sizes of *Bam*HI, *Eco*RI, and *Hin*dIII fragments detected by the Southern blot analysis of the mouse genomic DNAs (see panel **B**) are also shown below the map of each gene. The regions downstream from the arrows are highly homologous among the three genes, whereas the regions upstream from the arrows differ between the *rae1α* and the *rae1β* and *rae1γ* genes (see text). **(B)** Southern blot analysis of the mouse *rae1* genes. Mouse genomic DNAs (10 μg each) were digested with *Bam*HI (B), *Eco*RI (E), or *Hin*dIII (H), fractionated on 0.7% agarose gels, transferred onto nitrocellulose filters, and hybridized with genomic probes 1 to 4 (A). λDNA digested with *Hin*dIII was loaded as size markers. The results obtained with probe 3 are included in a foregoing paper (*5*).

genes contain six exons highly homologous to exons 4 to 9 of the *rae1α* gene (Fig. 1A). These DNAs contain one more exon in regions corresponding to intron 3 of the *rae1α* gene (Fig. 1A). We have not succeeded in isolating the full-length Rae1β and Rae1γ cDNAs, and we noticed that the 5′-end regions of the isolated Rae1β and Rae1γ cDNAs are extremely G-rich, *i.e.*, 34 of the 5′-end 60 nt of the cloned Rae1β and Rae1γ cDNAs are guanosines (57%), while the corresponding region of Rae1α cDNA contains 19 guanosines (32%) (data not shown). Without acquisition of full-length cDNAs, we have not been able to determine numbers and locations of exons in the *rae1β* and *rae1γ* genes. The restriction map of the region upstream from the middle of intron 1 of the *rae1α* gene differs from that of the corresponding regions of *rae1β*/*rae1γ* genes, as shown in Fig. 1A.

Southern Blot Analysis of rae1 Family Members—To confirm the presence of three different *rae1* genes in the mouse genome, Southern blot analysis was done using probes prepared from the cloned *rae1* genes (Fig. 1A). Representative results are summarized in Fig. 1B. Probe 1 prepared from the *rae1γ* gene detected only one band in each digest, *i.e.*, one 6.4-kb band in the *Bam*HI digest, one 5-kb band in the *Eco*RI digest, and one 5-kb band in the *Hin*dIII digest (Fig. 1B), thereby suggesting the absence in the *rae1α* gene of a DNA sequence hybridizable with this probe (Fig. 1A). Results obtained with probes 2, 3, and 4 support the presence of three different *rae1* genes in the mouse genome, *i.e.*, two bands each were detected in *Bam*HI digests with probe 2, in *Bam*HI and *Eco*RI digests

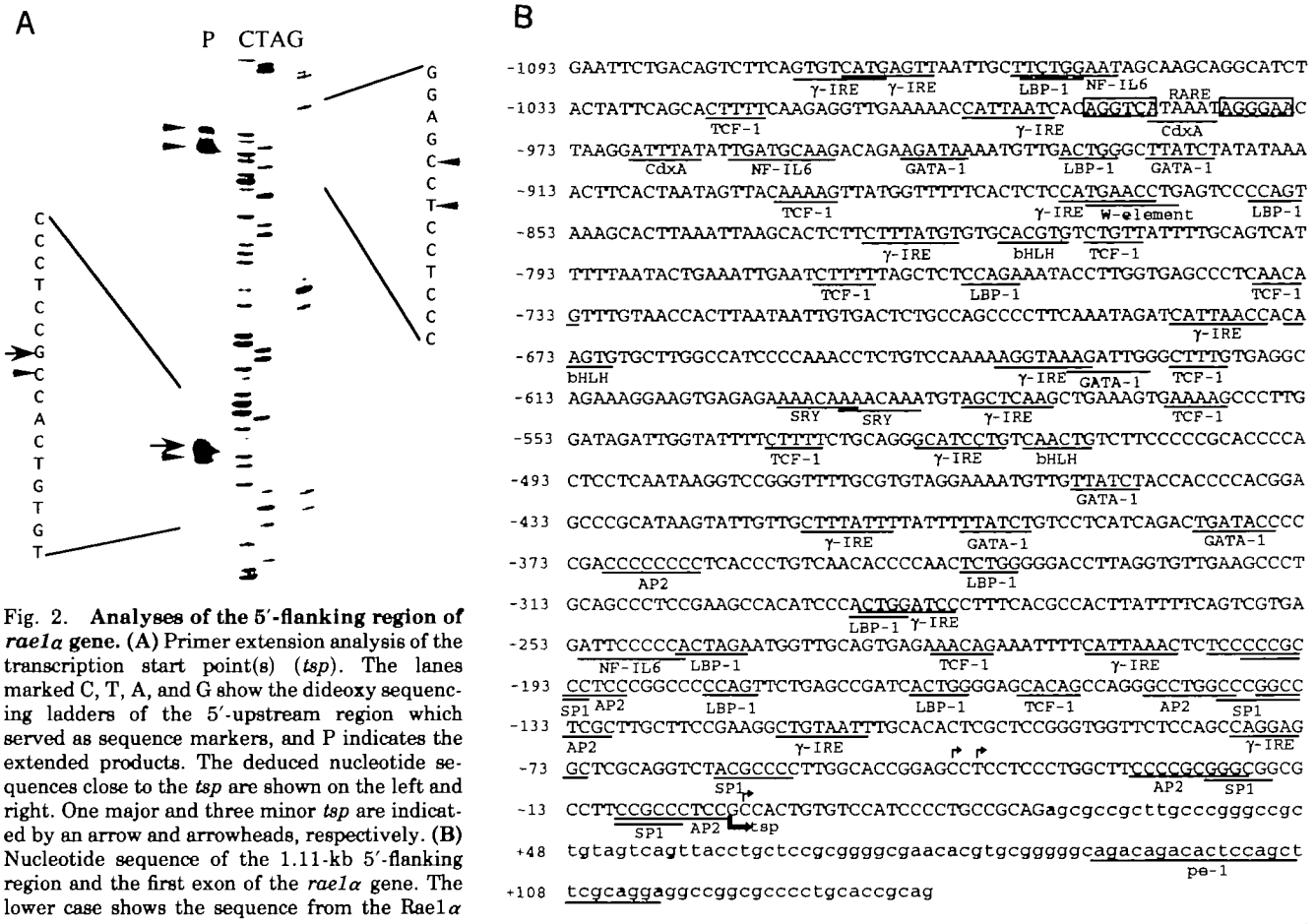

Fig. 2. **Analyses of the 5′-flanking region of *rae1α* gene. (A)** Primer extension analysis of the transcription start point(s) (*tsp*). The lanes marked C, T, A, and G show the dideoxy sequencing ladders of the 5′-upstream region which served as sequence markers, and P indicates the extended products. The deduced nucleotide sequences close to the *tsp* are shown on the left and right. One major and three minor *tsp* are indicated by an arrow and arrowheads, respectively. **(B)** Nucleotide sequence of the 1.11-kb 5′-flanking region and the first exon of the *rae1α* gene. The lower case shows the sequence from the Rae1α cDNA, the upper case shows that from the genomic DNA. The major and minor *tsp* are indicated by bold and thin arrows. The nucleotide corresponding to the major *tsp* is numbered as +1 and is 26 bp upstream from the 5′-end of the longest Rae1α cDNA (*5*). Potential binding sites for regulatory factors are underlined with names of the factors listed below the lines. Abbreviations are as follows: γ-IRE, γ-interferon responsive element (*18*); TCF-1, T-cell specific transcription factor-1 (*19*); LBP-1, long terminal repeat-binding factor-1 (*20*); NF-IL6, IL-6 responsive nuclear factors (*21*). The putative retinoic acid-responsive element (RARE) is marked by two boxes. The nucleotide sequence data reported here will appear in DDBJ, EMBL, and GenBank databases under accession number D85807.

with probe 3, and in *Hin*dIII digests with probe 4 (Fig. 1B). These results are in good agreement with restriction maps summarized in Fig. 1A.

Analyses of the 5′-Flanking Region of the rae1α Gene—Regulatory signals of the *rae1α* gene should be of interest, because this gene is inducible in F9 cells by RA, and is expressed exclusively in early mouse embryos (*5*). We first determined the transcription start point(s) (*tsp*) by primer extension using the pe-1 as a primer (Fig. 2B) and found four *tsp* close to the 5′-end of Rae109 cDNA (*5*) (Fig. 2A). We designated the one with the strongest signal as the major *tsp*, and the nt corresponding to this site as +1 of the *rae1α* gene (Fig. 2, A and B).

To elucidate the regulatory sequences, we subcloned and sequenced the 1.1-kb *Eco*RI/*Sma*I fragment corresponding to the 5′-flanking region of *rae1α* (Fig. 1A, EI19); the sequence is shown in Fig. 2B. Neither TATA-like nor CCAAT-like boxes were found in this region. Computer analysis revealed the presence of several potential Sp1 and AP2 binding sites and potential sites for many other regulatory factors (Fig. 2B). Interestingly, we found in this region a putative retinoic acid-responsive element (RARE) of the DR5 type (*22*) (Fig. 2B).

Chromosomal Assignment of the rae1 Family Members—Since mouse MHC class I genes have already been mapped on chromosome 17 (*23*), we mapped the chromosomal localization of the *rae1* family members by R-banding fluorescence *in situ* hybridization (FISH), using as a probe a 3.0-kb genomic fragment (see Fig. 1A, EIX2). As shown in Fig. 3, the signals were localized on the R-positive A4 band of mouse chromosome 10 (*23-25*). The probe used in this experiment is highly homologous to all three *rae1* genes. Hence, the present FISH data suggest that the three *rae1* family genes are localized on A4 band of mouse chromosome 10 (*14, 15*).

Zoo Blot Analyses of the rae1 Family Members—Since the deduced RAE1 proteins show weak homology with MHC class I molecules, we asked if the *rae1* family members had been conserved during evolution. High molecular weight DNAs were prepared from human, rat, mouse, chicken, *Xenopus*, *Drosophila*, yeast, and *E. coli*, digested by *Eco*RI, and subjected to Southern blot analysis under conditions of high and low stringency, respectively (Fig. 4). Rae1 cDNA (*5*) and part of the mouse MHC class I gene (*12*) were used as probes. Although these probes detected several discrete bands in mouse (Fig. 4a), the

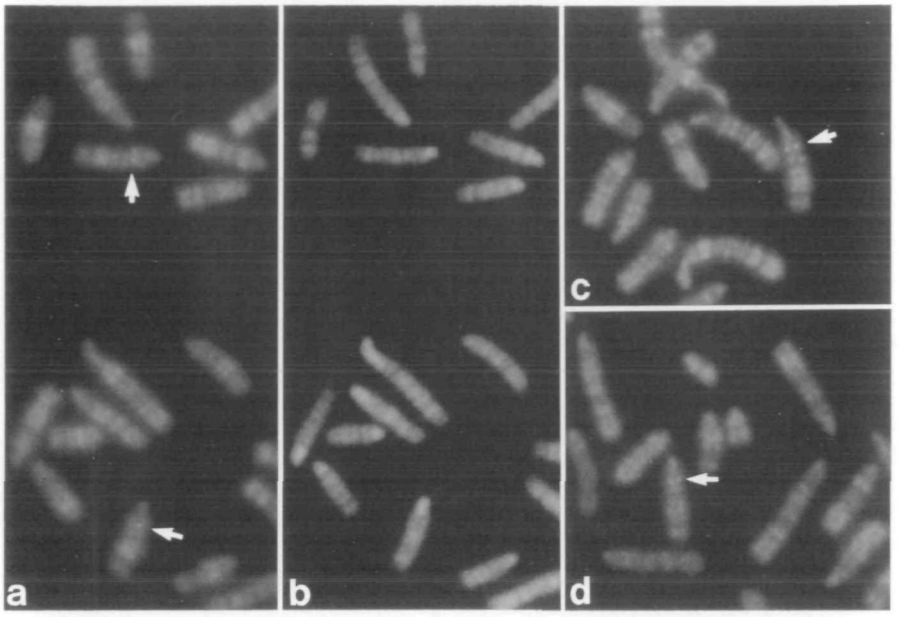

Fig. 3. **Chromosomal localization of *rae1* family members on R-banded chromosomes.** Left panel: a representative R-banded metaphase spread after *in situ* hybridization with *rae1* genomic probe EIX2 (Fig. 1A). The arrows point to hybridization signals of both alleles on chromosome 10. Right panel: a G-banded diagram of the same metaphase spread. The R-banded chromosome slides were denatured and dehydrated in 70-85-100% ethanol series. The probe was labeled by nick translation with biotin 16-dUTP (Boehringer Mannheim) and hybridized on the denatured slide at 37°C overnight. After washing, the slide was incubated under a coverslip with fluoresceinated avidin at a 1:500 dilution in 1% BSA/4×SSC, then stained with 0.75 μg/ml propidium iodide. Excitation wavelengths of 450-490 nm (Nikon filter set B-2A) and near 365 nm (UV-2A) were used for the observation of R-banding and G-banding patterns, respectively (*14, 15*). The metaphase spreads were photographed with Nikon B-2A **(a, c, d)** and UV-2A **(b)** filters. R-band and G-band patterns are demonstrated in **(a, c, d)** and **(b)**, respectively.

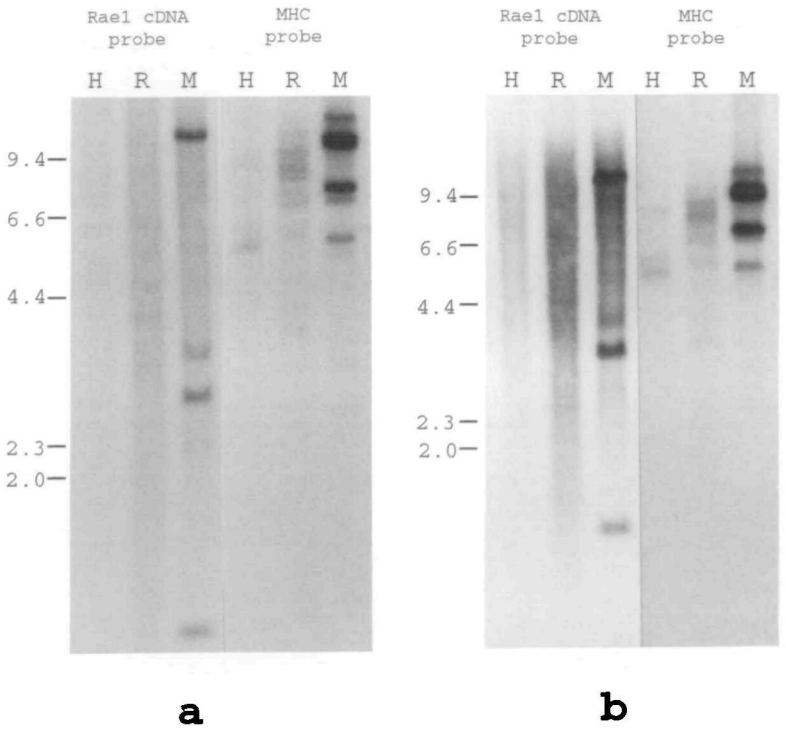

Fig. 4. **Zoo blot analysis of the *rae1* family members.** High molecular weight DNAs extracted from human peripheral polymorphocytes, rat and mouse liver were digested with *Eco*RI, separated on an 0.8% agarose gel and transferred to nylon membrane. These blots were hybridized with ^{32}P-labeled Rae1 cDNA or mouse MHC class I genomic DNA probe. Symbols are as follows: H, human; R, rat; M, mouse. **(a)** Hybridization and washing were done under conditions of high stringency, as for Fig. 1B. **(b)** Hybridization (6×SSC, 55°C) and washing (2×SSC, 55°C) were done under conditions of low stringency.

Rae1 probe detected no bands in chicken, *Xenopus*, *Drosophila*, yeast, and *E. coli* (data not shown), rat and human, even under low conditions of stringency (Fig. 4b). On the other hand, the mouse MHC class I probe detected several bands in rat and human, under conditions of high and low stringency (Fig. 4, a and b). Therefore, *rae1* family members probably diversified rapidly during evolution.

In Situ Hybridization Analysis of Rae1 mRNAs in Early Mouse Embryos—By Northern blot analysis, the Rae1 mRNAs were not detected in adult mice, but were detected exclusively in early mouse embryos (*5*). To determine the stages and sites of *rae1* expression, we examined expression patterns of *rae1* genes in 9, 10, and 11 d.p.c. embryos by whole-mount *in situ* hybridization. In a 9 d.p.c. embryo, *rae1* expression was detected throughout the embryo including the regions covering the frontal part of brain, branchial arches, heart, and limb buds (Fig. 5a). In a 10 d.p.c. embryo, strong expression of *rae1* was noted in brain,

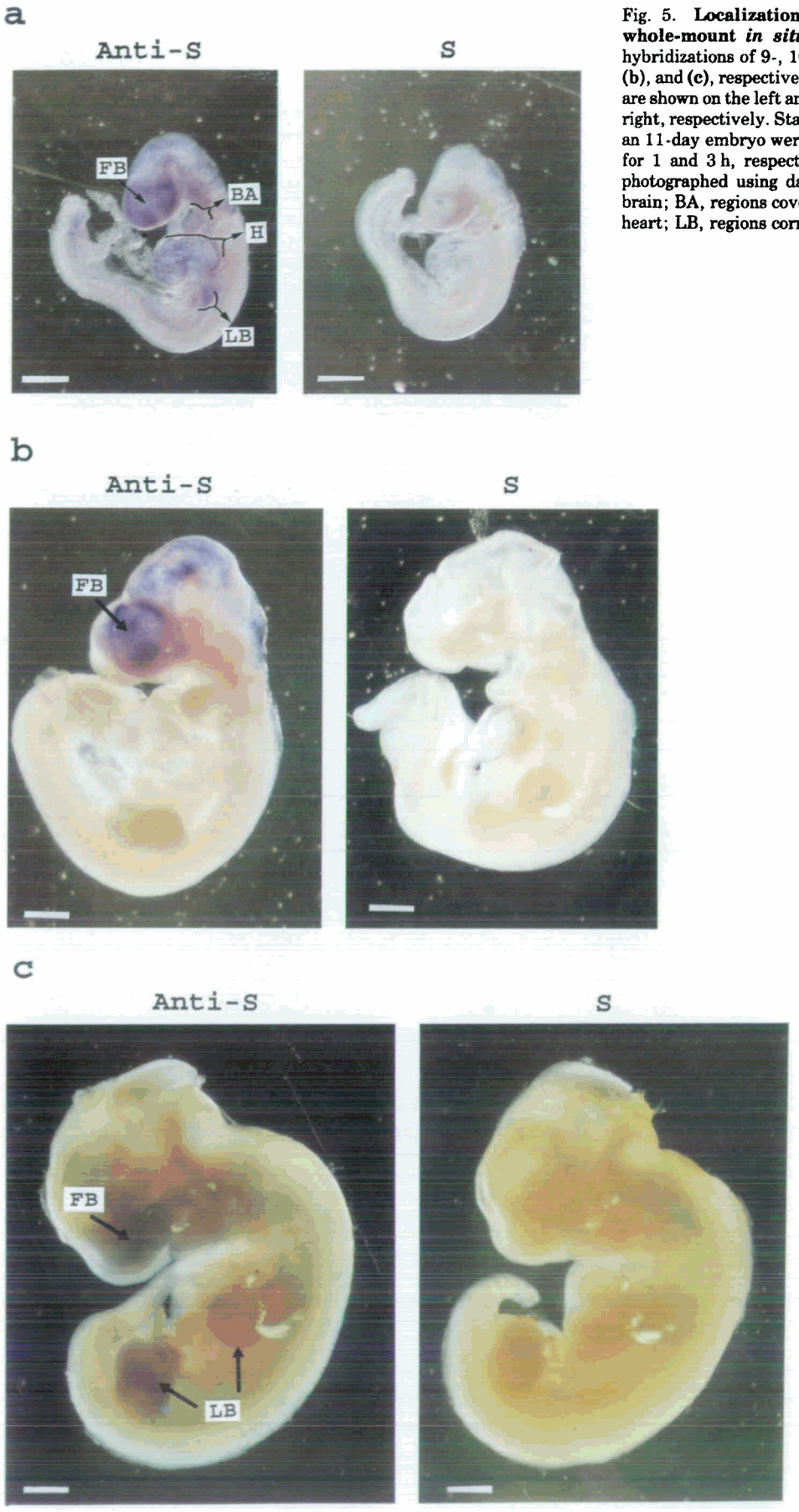

Fig. 5. **Localization of Rae1 mRNAs in mouse embryos by whole-mount *in situ* hybridizations.** Whole-mount *in situ* hybridizations of 9-, 10-, and 11-day embryos are shown in **(a)**, **(b)**, and **(c)**, respectively. The results of antisense probe **(Anti-S)** are shown on the left and those of sense probe **(S)** (control), on the right, respectively. Staining of 9- and 10-day embryos and that of an 11-day embryo were allowed to proceed at room temperature for 1 and 3 h, respectively, and representative embryos were photographed using dark-field microscopy. FB, frontal part of brain; BA, regions covering branchial arches; H, region covering heart; LB, regions corresponding to limb buds. Bars: 400 μm.

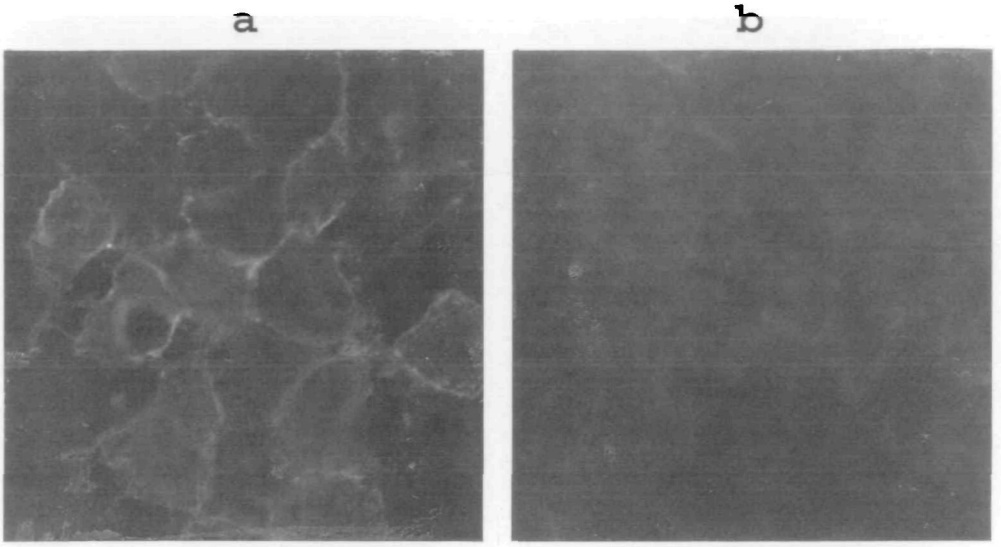

Fig. 6. **Effect of PI-PLC treatment on cellular localization of RAE1 proteins.** (a) RAE1-Myc fusion proteins were detected on surfaces of transfected F9 cells by using a monoclonal antibody against the human c-Myc epitope. (b) Cells were preincubated with PI-PLC and most immunoreactivities detected in (a) were removed after PI-PLC treatment.

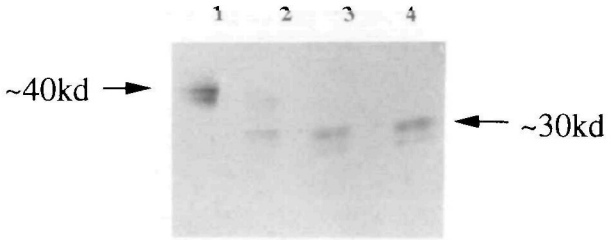

Fig. 7. **Glycosylation of RAE1 proteins.** RAE1 proteins were analyzed by immunoblot analysis. Lane 1: F9-Rae1 transfectants; lane 2: F9-Rae1 transfectants cultured in the medium including 0.5 μg/ml of tunicamycin; lane 3: F9-Rae1 transfectants cultured in the medium including 1.0 μg/ml of tunicamycin; lane 4: F9-Rae1 transfectants cultured in medium including 2.0 μg/ml of tunicamycin.

especially in the regions corresponding to its frontal part (Fig. 5b). In an 11 d.p.c. embryo, the *rae1* expression was weaker than that observed in 9 and 10 d.p.c. embryos. Expressions in the regions corresponding to the frontal part of brain and to limb buds were evident only after a longer period of staining (Fig. 5c). These findings show that the *rae1* genes are transcribed in early embryos, predominantly in the brain, and that the expression of *rae1* is regulated in a stage- and space-specific manner.

RAE1 Attachment to the Cell Surface Is Sensitive to Phosphatidylinositol-Specific Phospholipase C in F9 Cells—We considered the possibility that the hydrophobic C-terminus of the deduced RAE1 protein (*5*) fits the consensus signal sequences for linkage to a GPI anchor and accounts for the association of RAE1 proteins with the cell surface (*26, 27*). To test this possibility, F9 cells were transfected with a Rae1-Myc expression vector containing a DNA fragment encoding 10 amino acids of the human c-Myc protein, within the reading frame of Rae1 cDNA (*5*). The RAE1-Myc fusion protein was immunocytochemically detected on the surface of the transfected F9 cells by making use of a monoclonal antibody against the human c-Myc epitope 9E10 (*5, 29*) (Fig. 6a). Cells expressing the RAE1-Myc fusion protein were then treated with phosphatidylinositol-specific phospholipase C (PI-PLC) (*5, 28*). We concluded that cell surface RAE1-Myc proteins are attached to the cell membrane *via* GPI linkage, because almost all of the immunoreactivities were removed from cell surfaces after the PI-PLC treatment (Fig. 6b).

RAE1 Proteins Are Glycosylated—The deduced RAE1 proteins contain five potential *N*-linked glycosylation sites and eight possible *O*-linked glycosylation sites (*5*), suggesting that RAE1 proteins are heavily glycosylated cell surface proteins. We estimated from immunoblot analysis that RAE1 proteins are around 40 kDa (Fig. 7), although the predicted sizes from amino acid sequences are less than 25 kDa (*5*). The size of RAE1 proteins synthesized in F9 cells decreases to less than 30 kDa following treatment with tunicamycin (Fig. 7). These data support the notion that RAE1 proteins are heavily glycosylated proteins.

DISCUSSION

Three genes corresponding to the three Rae1 cDNAs were isolated from 129/SVJ and, although most areas of these three are highly homologous, the restriction map of the 5′-end region of the *rae1α* gene differs from that of the corresponding regions of the *rae1β* and *rae1γ* genes (Fig. 1A). This observation is consistent with the finding that *rae1α* is under different regulatory control from *rae1β* and *rae1γ*: the *rae1α* gene is predominantly expressed in RA-induced F9 cells, but at a low level in mouse embryos; but *rae1β* and *rae1γ* genes are expressed at low levels in RA-induced F9 cells, but at relatively high levels in mouse embryos (*5*).

We reported that RAE1 proteins share a weak homology with MHC class I molecules (*5*). In the present work, we found that DNA fragments hybridizable with Rae1 cDNA are not detectable in rat and human genomes (Fig. 4). Hence, Rae1 family members have apparently evolved rapidly, as is the case for MHC class I genes. Although these observations do suggest a possible link between functions of RAE1 proteins and MHC class I molecules, we have no data directly indicating what the functions of RAE1 proteins are.

The Rae1 cDNAs were isolated as RA-inducible clones, and *rae1* genes are expressed exclusively in the early mouse embryos, predominantly in the embryonic brain (Fig. 5). The developing or regenerating nervous system

presumably requires two types of factors, one that promotes growth and survival, and one that provides guidance in the establishment of neuronal pathways. Factors not freely diffusible are good candidates for the latter. Recently, ELF-1 (*30*) and RAGS (*31*) were identified as members of a ligand family for the Eph-like receptors and it was suggested that they may participate in guidance of retinal ganglion cell axons. All the members of this ligand family are membrane-bound *via* either a GPI-linkage or a transmembrane domain (*32*). Membrane attachment is not only necessary for efficient activation of receptors by ligands, it is also essential for normal functions (*33*). All family members share a homologous extracellular domain with four conserved cysteine residues (*32*). The RAE1 proteins are attached to the membrane by a GPI anchor (Fig. 6) and are probably heavily glycosylated (Fig. 7). Although RAE1 proteins share no significant sequence homology with Eph-ligands, they do share an extracellular domain of similar size with four conserved cysteine residues. These features suggest that RAE1 proteins may play similar roles to Eph-ligands. Isolation and characterization of proteins interacting with RAE1 proteins should aid in elucidating their function(s).

The RA-inducibility and the putative RARE sequence in the 5′-flanking region of the *rae1α* gene suggest that RAE1α protein is a mediator of RA actions in some embryonic tissues. LERK-2, an Eph-ligand, was also identified as an RA-inducible cDNA clone from RA-induced P19 cells (*34*). LERK-2 and its receptor are expressed in the forebrain as well as in the other areas of the embryonic brain (*34*). Analysis of the developmental roles of RAE1α and LERK-2 and how they are regulated by RA *in vivo* are expected to lead to a better understanding of how retinoids regulate development.

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