Syncytin-A and syncytin-B, two fusogenic placenta-specific murine envelope genes of retroviral origin conserved in Muridae

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Recently, we and others have identified two human endogenous retroviruses that entered the primate lineage 25–40 million years ago and that encode highly fusogenic retroviral envelope proteins (syncytin-1 and -2), possibly involved in the formation of the syncytium of syncytiotrophoblast layer generated by trophoblast cell fusion at the materno–fetal interface. A systematic in silico search through human genome databases presently identifies two fully coding envelope genes, present as unique copies and unrelated to any known murine endogenous retrovirus, that we named syncytin-A and -B. Quantitative RT-PCR demonstrates placenta-specific expression for both genes, with increasing transcript levels in this organ from 9.5 to 14.5 days postcoitum. In situ hybridization of placenta cryosections further localizes these transcripts in the syncytiotrophoblast-containing labyrinthine zone. Consistently, we show that both genes can trigger cell–cell fusion in ex vivo transfection assays, with distinct cell type specificities suggesting different receptor usage. Genes orthologous to syncytin-A and -B and disclosing a striking conservation of their coding status are found in all Muridae tested (mouse, rat, gerbil, vole, and hamster), dating their entry into the rodent lineage ~20 million years ago. Together, these data strongly argue for a critical role of syncytin-A and -B in murine syncytiotrophoblast formation, thus unraveling a rather unique situation where two pairs of endogenous retroviruses, independently acquired by the primate and rodent lineages, would have been positively selected for a convergent physiological role.

Endogenous retroviruses (ERVs) are present in the genome of all vertebrates (1–3). They are most probably the genomic tracers of germ-line infections by exogenous retroviruses having taken place during evolution. Over time, increase in their copy number has occurred, at least for some of them, via retrotransposition or germ-cell reinfecction. This generated multigenic families containing a few to several hundred elements and now accounting for a substantial fraction of the genome [8% for the human (4) and 10% for the murine (5) genomes]. In mice, eight families of ERVs, with a structure close to the integrated proviral form of exogenous retroviruses (gag-, pol-, and env-related regions bordered by two LTRs), have been described. Those with close exogenous relatives include type B proviruses related to the murine mammary tumor virus (6) and type C proviruses related to the Moloney murine leukemia virus (MLV), with the endogenous MLV, MuRRS, MuRVY, GLN, MuERV, and MmERV family members (1, 7, 8).

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Abbreviations: Myr, million years; ERV, endogenous retrovirus; MLV, murine leukemia virus; dpc, days postcoitum; SU, surface; TM, transmembrane.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY849973–AY849976 for the mouse, rat, vole, and hamster syncytin-A, respectively; and AY849897–AY849898 for the mouse, rat, gerbil, vole, and hamster syncytin-B, respectively).

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that of the human syncytin genes. Accordingly, at least in two cases of animal evolution, i.e., in primates and rodents, a pair of ERVs would have been independently acquired and put to work for a common physiological role.

Materials and Methods
Database Screening and Sequence Analyses. Retroviral envelopes were searched by using the BIOMOTIF program (www.lpta.univ-montp2.fr/software.html) and the degenerate universal CKS17u consensus motif in ref. 17. Multiple alignments were carried out by using the CLUSTALW program (www.infobiogen.fr). Phylogenetic trees were constructed from alignments by using the neighboring-joining program within CLUSTALW and were viewed with the NPILOT program. Repeat sequences were identified by using the REPEAT-MASTER server (www.repeatmasker.org). Homologous sequences were searched by using the BLAST programs at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST) and at the Ensembl (www.ensembl.org) sites. Nonsynonymous vs. synonymous substitution ratios (Ks/Ka) were calculated by using the SNAP.PL program at www.hiv.lanl.gov.

Real-Time Quantitative PCR, RT-PCR, and Northern Blot Analyses. C57BL/6 mice, including pregnant females at different gestational stages [morning of vaginal plug considered as 0.5-day postcoitum (dpc)], were from Janvier (Le Genest-St-Ise, France). Total RNA was extracted from frozen organs (pooled from at least two mice) with the RNeasy RNA isolation kit (Qiagen, Valencia, CA). Reverse transcription and quantitative PCR were performed as ref. 18, with primers TACTCTCTGCCCCGATAGATGA and CCGTTTTCTTAAACAGTGGTG for syncytin-A and CCACCCATACGGTCAAA and GTTATACGAGGTCGCGAAG for syncytin-B. Structure of the syncytin transcripts was determined by RT-PCR (primer sequences available upon request). Northern blot analysis was performed as in ref. 19, using syncytin-A or -B 32P-labeled full-length env gene probes.

In Situ Hybridization. Placenta tissues (14.5 dpc) were processed for serial cryosections (10 μm thick). The antisense and sense riboprobes were in vitro transcribed with T7 RNA polymerase by using digoxigenin-11-UTP (Roche Applied Science, Indianapolis) and the syncytin-A and -B full-length env genes cloned in the two orientations into PGEM-T (Promega) as templates. Cryosections were postfixed at 4°C in 1% paraformaldehyde for 5 min, hybridized at 65°C overnight with the riboprobes, and further incubated with antidigoxigenin antibodies overnight at room temperature. The signal was amplified by using the Vectastain ABC kit (Vector Laboratories) coupled to the Tyramide Signal Amplification system (NEN Life Sciences). Histological examination was performed after DAPI nuclear staining by using a Zeiss Axioskop microscope.

Envelope Gene Expression Vectors. The A-Rless hypervarigenic mutant amphotropic MLV envelope expression vector and the pHCMV-G plasmid are in ref. 14. The syncytin-A and -B envelope expression vectors were constructed as follows: syncytin-A was PCR amplified from C57BL/6 genomic DNA, using the proofreading Platinum Pfx DNA polymerase (Invitrogen) for 30 cycles, and syncytin-B was PCR-amplified from the corresponding C57BL/6 bacterial artificial chromosome (AC134575) DNA, using the proofreading Pfu/Turbo Hotstart polymerase (Stratagene) for 15 cycles (primer sequences available upon request); the PCR products were cloned into the pHCMV-G vector cut with EcoRI and blunt-ended.

Cell Lines and Fusion Assay. The murine MCA205 fibrosarcoma, WOP fibroblast, and LOK kidney cells (gift from S. Fichelson, Institut Cochin de Genétique Moleculaire, Paris), the rat 208F fibroblast cells, hamster BHK-21 kidney cells, canine MDCK kidney cells, feline G355-5 astrocyte cells, monkey VERO kidney cells, the human TE671 rhabdomyosarcoma, 293T embryonal kidney, and HeLa carcinoma cells, were grown in DMEM with 10% FCS; the Chinese hamster ovary cells were grown in DMEM/F12K (1:1), with 7% FCS. For fusion assays, cells were transfected by using Lipofectamine (Invitrogen) and 1–2 μg of DNA for 5 × 105 cells, except for 208F, 293T, and TE671 cells, which were transfected by using calcium phosphate precipitation (Invitrogen, 5–20 μg of DNA for 5 × 105 cells). Plates were inspected for cell fusion 24–48 h after transfection. Syncytia were visualized by using May–Grünwald and Giemsa staining (Sigma) and the fusion index calculated as [(N − S)/T] × 100, where N is the number of nuclei in the syncytia, S is the number of syncytia, and T is the total number of nuclei counted.

DNAs and Southern Blot Analyses. Bacterial artificial chromosome clones were from BACPAC Resources (Oakland, CA). Genomic DNAs were extracted from animal tissues or blood cells. C57BL/6 mouse (Mus musculus), rat (Rattus norvegicus), gerbil (Meriones unguiculatus), and hamster (Mesocricetus auratus) were from Janvier. Guinea pig (Cavia porcellus) DNA was extracted from peripheral blood leukocytes (Charles River Laboratories). Vole (Clethrionomys glareolus) tissue was a gift from F. Catzeflis (Institut des Sciences de l’Evolution, Montpellier, France). Squirrel (Spermophilus beecheyi) and woodchuck (Marmota monax) tissues were a gift from M.-A. Buendia (Institut Pasteur, Paris). For Southern blot analyses, membranes were hybridized overnight at 65°C in 7% SDS/1 mM EDTA/0.5 M Na2HPO4, using 32P-labeled syncytin-A or -B full-length env probes, and washed at 65°C under low-stringency conditions (2× SSC/0.1% SDS for 15 min and 1× SSC/0.1% SDS for 10 min) before x-ray film exposure.

Characterization of the Syncytin-A and -B Genes from Rodents. The orthologous genes and flanking sequences were sorted out step by step by a series of low-stringency direct PCRs carried out for 30–45 cycles (10 sec at 94°C, 30 sec at 50°C, 1–6 min at 68°C), using 100 ng of genomic DNA, the Expand long-template enzyme mix (Roche Applied Science); in some cases, inverted PCRs were performed as described in ref. 20 by using genomic DNA first digested by appropriate restriction enzymes and then self-ligated, and pairs of primers in opposite orientation. For unambiguous sequence determination, the entire syncytin-A and -B orthologous genes were finally amplified from 100 ng of genomic DNA for 25 cycles, using the Expand long-template enzyme mix (Roche Applied Science) and primers designed from the characterized flanking sequences, and sequencing was performed on the bulk of the PCR products without cloning (sequences have been deposited in the GenBank database; accession nos.AY849973–AY849981).

Results
Identification of Two Previously Uncharacterized Retroviral Envelope Genes in the Mouse Genome. To identify env genes within the mouse genome, we used a method previously devised to screen the whole human genome for such genes (17). Basically, it makes use of the degenerate CKS17u consensus motif, associated with the immunosuppressive domain of retroviral envelopes and designed to match the majority of envelope genes of exogenous and endogenous origin (17). Murine sequences from the GoldenPath database and from the nr (nonredundant) and HTGS (high-throughput genomic sequences) divisions of the GenBank database (release 132) were screened with this motif by using the BIOMOTIF program. A 2-kb region encompassing 1,500 bp upstream and 500 bp downstream of the CKS17u motif was extracted from positive sequences, and only those regions containing an ORF > 1.6 kb were considered further. Selected ORFs (~200), plus representatives of known endogenous and exogenous retroviral env genes, were then aligned and phylogenetic trees constructed to identify and classify the sequences that were sorted out. All of them (except two; see below) were found to belong to multicopy families of type C origin, with about one-half as MmERV (8), one-quarter as endogenous
MLV (21), and one-quarter as GLN sequences (providing, by the way, the first description of GLN fully coding env genes in the mouse genome; ref. 22). Note that IAPE and endogenous MMTV env genes were not sorted out by the screen, because they are devoid of the CKS17 motif. Interestingly, two previously uncharacterized env sequences (AF289664 and AC134575), not belonging to any known murine ERV family and each present as a unique copy, were identified with that screen. A phylogenetic analysis (Fig. 1) places them within the enlarged HERV-F/H20862H family, also containing the human syncytin-1 and -2 env genes, but in branches distinct from the latter. These two newly identified murine env sequences were subsequently named syncytin-A and -B because of their functional properties closely related to those of the human syncytin genes (see below).

Amino Acid Sequence and Genomic Organization of the Syncytin-A and -B Genes. Analysis of syncytin-A and -B amino acid sequences together with their hydrophobic profiles (Fig. 2), discloses all of the characteristic features of retroviral envelopes (reviewed in ref. 23), with a canonical cleavage site (consensus: R/H20862K-N-R/H20862K-R; ref. 24) between the surface (SU) and transmembrane (TM) moieties of the proteins and the presence of hydrophobic domains corresponding to the fusion peptide and the TM domain. A CWVC domain, involved in the interaction between the SU and TM moieties in retroviral envelopes (25), and an immunosuppressive domain (26) can also be identified. Amino acid sequence alignment shows 67% identity between the two genes, with maximum similarity in the TM region (data not shown). Syncytin-A and -B loci can be assigned to chromosomal positions 5gG2 and 14qD1, respectively. Analysis of the genomic sequences surrounding the env ORFs discloses extremely degenerate proviral structures (Fig. 3), with only short regions with homology to pol and the presence of solitary ERV and MaLR LTRs, which could serve for initiation and termination of syncytin transcription (see below), as well as of repetitive B1, B2, and LINE elements. Finally, the regions flanking the syncytin-A and -B proviral structures are not orthologous (no sequence similarity), consistent with distinct retroviral integration events.

Placenta-Specific Expression of the Syncytin-A and -B Genes. Syncytin-A and -B transcript levels in mouse tissues were investigated by real-time quantitative RT-PCR, by using primer pairs designed and demonstrated to be specific for each gene. As illustrated in Fig. 4A, syncytin-A and -B are expressed essentially in the placenta, with only few organs above background level. Analysis of embryos and embryonic annexes (yolk sac, amnion, and placenta) at different developmental stages (from 7.5 to 16.5 dpc) further supports placenta-specific expression of syncytin-A and -B and reveals strong induction of both genes in this organ between 9.5 and 14.5 dpc (Fig. 4B). Northern blot analysis of placenta RNAs discloses a single transcript of 3.5 kb for syncytin-A and two transcripts of 3.7 and 3 kb for syncytin-B (Fig. 4C). Their structure was partially characterized by RT-PCR and in silico searches throughout EST and cDNA databases (Fig. 3).
revealed multiple alternative splicing for both env transcripts, reminiscent of the spliced subgenomic env mRNAs of retroviruses and consistent with a retroviral origin of these genes, and two alternative polyadenylation signals for syncytin-B, all features compatible with the observed size of the transcripts.

Localization of Syncytin-A and -B Expression in the Placenta by in Situ Hybridization. In situ hybridizations (Fig. 5) were performed on 14.5 dpc placenta cryosections (see scheme of the placenta tissue in Fig. 5 and ref. 27), using digoxigenin-labeled probes. Syncytin-A and -B expression is observed all over the labyrinthine zona (Fig. 5 B and C), with labeling detected neither in the spongiotrophoblastic and giant cell zona nor in the maternal decidua (not shown); no labeling can be detected in control hybridizations using a sense syncytin-A probe (Fig. 5 A). The murine labyrinthine zona mainly contains spongiotrophoblasts formed by fusion of mononuclear trophoblastic cells, arranged as barriers between the maternal and fetal blood circulation (see scheme). Higher magnification of the labeled labyrinthine zona (Fig. 5 D and E) provides indication for syncytin-A and -B expression at the level of spongiotrophoblasts, with the labeling encompassing structures with several nuclei. Labyrinth-specific expression of both genes is consistent with the kinetics of accumulation of syncytin-A and -B transcripts as revealed by quantitative RT-PCR (Fig. 4B), which follow the kinetics of development of the placenta labyrinthine layer, taking place from 9.5 to 15.5 dpc (27, 28).

Syncytin-A and -B Are Fusogenic, with Different Receptor Usage. To determine whether the syncytin-A and -B gene products have fusogenic properties, expression vectors for the corresponding proteins were used to transfect a series of cells of distinct origins, which were thereafter screened for the presence of syncytia (Fig. 6). The highly fusogenic A-Rless MLV mutant envelope (29) and syncytin-A in antisense orientation were used as controls. As illustrated in Table 1, cell–cell fusion can be observed for several murine, human, and monkey cell lines with syncytin-A, whereas syncytin-B is found to be fusogenic only with the canine MDCK kidney cells, suggesting a rather rarely distributed receptor for the latter. That the fusogenic potencies of syncytin-A and -B proteins are different is strongly in favor of different receptor usage for these two envelope proteins. Yet, the observed cell specificity patterns for fusion are distinct from those of the human syncytin-1 and -2 proteins (14) and do not allow any prediction as to the nature of the receptors involved.

Syncytin-A and -B env Gene Acquisition and Conservation During Rodent Evolution. To tentatively identify syncytin-A and -B orthologs in other species, DNAs from eight rodents were digested with DraI, which does not cut in the murine syncytin-A and -B env genes, and examined by Southern blot with syncytin-A or -B probes under low-stringency conditions. As illustrated in Fig. 7, hybridizing fragments are detected for both genes in Muridae only (mouse, rat, gerbil, vole, and hamster), with no signal for the squirrel, wood-chuck, and guinea pig. In the rat, the DraI-restricted syncytin-A and -B bands display identical sizes, consistent with sequencing data (see below). According to the zoo-blot, the date of entry of the syncytin-A and -B genes into the rodent lineage should be before speciation of Muridae, ~20 Myr ago (30). BLAST searches performed using rat genome databases provided the sequence of the syncytin-A (GenBank accession no. AC091000) and the syncytin-B (contig RNOR03237248 in Ensembl and XM.224362 in GenBank) orthologs, as fully coding genes. To identify the other Muridae orthologs, sets of primers based on regions conserved between the
structures with several nuclei. Nuclei were stained in blue by using DAPI.

Fig. 5. In situ hybridization of 14.5 dpc mouse placenta for syncytin-A and -B expression. Scheme of a mouse mature placenta cross section: the labyrinthine zona (LZ) is the site of exchange between the maternal blood (Mb) and the fetal blood vessels (Fbv) and contains mononuclear trophoblast cells (MT) that terminally differentiate, by cell fusion, into syncytiotrophoblasts (ST) directly lining the fetal blood vessels; in Muridae, both cell types are arranged as a three-layered barrier (two syncytial and one mononuclear layer, not resolved) between the maternal and fetal blood circulation; the spongiotrophoblast zona with the spongiotrophoblast cells (SPT) and the outermost layer with the trophoblast giant cells (GC) (producing hormone/growth factors) overlie the labyrinthine zona. (A) Negative control: tissue section probed with a digoxigenin-labeled sense syncytin-A riboprobe. (B and C) Tissue sections probed with digoxigenin-labeled antisense syncytin-A and -B riboprobes, respectively, disclosing hybridization signals only within the labyrinthine and spongiotrophoblastic zona. (D and E) Magnification of the labyrinthine zona in B and C, with the arrowheads pointing to labeled structures with several nuclei. Nuclei were stained in blue by using DAPI. A–C, ×100; D and E, ×400.

Discussion

The present search for complete env genes in mouse genome databases has identified numerous sequences of known highly reiterated ERV families, together with two fully coding env genes, present at a single copy, and that we named syncytin-A and -B. These mouse genes display fusogenic activities when expressed in transfected cells, with differences in the cell types prone to fusion, indicating differences in receptor usage. Both genes are specifically expressed in the placenta and, more precisely, in the syncytiotrophoblast-containing labyrinthine zona. These features are closely related to those of the human syncytin-1 and -2 genes, suggesting a role in trophoblast fusion and placenta physiology. Yet the murine and human genes are not orthologs, with entry dates ~20 Myr ago for the murine genes and 25–40 Myr ago for the human genes, i.e., after the speciation of rodents and primates. Phylogenetic analyses (Fig. 1), disclosing distinct branching of the human and murine syncytin genes, as well as evidence that they are not syntenic, further support these conclusions. Analysis of the syncytin-A and -B genomic loci finally indicates that the two genes were acquired from two ancient retroviral integration events, with recognizable vestiges of proviral structures (conservation of short pol-related sequences and generation of multiple spliced env transcripts). These degenerate structures, much more conserved for the human syncytin-1 and -2 loci, are reminiscent of the severely altered Fv4 murine locus (10), a feature that may be explained by the high rate of nucleotide substitution in rodents (31).

Table 1. Fusion host range of the syncytin-A and -B envelopes genes.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell</th>
<th>Syncytin-A</th>
<th>Syncytin-B</th>
<th>A-Rless</th>
<th>Control</th>
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<tr>
<td>Mouse</td>
<td>MCA205</td>
<td>+</td>
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<td>+++</td>
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<td>208F</td>
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Experimental conditions were the same as in Fig. 6, with the hyperfusogenic-mutant amphotropic MLV envelope (A-Rless) used as a reference. Fusion indices were determined as indicated in Materials and Methods, with the following scale: −, <5; +, 5–20; + +, 20–70; ++ +, >70.
The syncytin-A and -B genes display remarkably similar patterns of expression, both quantitatively, with identical kinetics of transcript accumulation during placenta development, and qualitatively, with a similar localization of the transcripts in the syncytiotrophoblast-containing labyrinthine zone. The presence of two syncytin genes with closely related and possibly redundant properties, as also observed for the human syncytin genes, is intriguing. One possibility is that conservation of env genes recognizing two distinct receptors is a safeguard for the host. Another hypothesis could be that cooperation between the two genes is required for an efficient fusion process. Additional studies are required to clarify this point. Among factors known to be critical for in vivo syncytiotrophoblast formation (32, 33), the glial cells missing-1 (Gm1) transcription factor is of special interest, because its pattern of expression is remarkably similar to that of syncytin-A and -B (28). In addition, Gm1-deficient mice display a failure in labyrinth formation and lack syncytiotrophoblasts (34, 35). Interestingly, it was shown that human syncytin-1 expression can be activated by the human Gm1 factor (36), and several putative Gm1-binding sites can be identified in the murine syncytin-A and -B loci (A.D., unpublished data). One could therefore hypothesize that syncytin-A and -B expression in the placenta is induced by Gm1 and that both the human and murine syncytin genes are similarly regulated.

An interesting result of the present study is the conservation of the syncytin-A and -B genes with a fully coding status over 200 Myr of rodent evolution, since their entry into the Muridae branch. Consistently, Muridae and non-Muridae rodents display well established differences regarding the structure and number of syncytiotrophoblast layers at the materno–fetal interface: although most rodents have a one-layered syncytiotrophoblast barrier (monochorial placenta), a three-layered structure, with one cellular and two syncytiotrophoblast layers (trichorial placenta), has emerged in Muridae (ref. 37 and Fig. 7). One could therefore hypothesize, provided that all syncytin orthologs are fusogenic, that these phenotypic changes result from the retrovirus-mediated acquisition of the syncytin-A and -B genes in Muridae, which would have given a selective advantage to the host. Yet, the presence of placenta syncytial cells in most other rodents suggests that fusogenic gene(s) did exist before the capture of the syncytin genes, but we know nothing about their nature or subsequent evolution in rodents. Indeed, placenta syncytial cells have originated on multiple independent occasions during mammalian evolution, being observed for example in Artiodactyla, but not in pigs or camels, in Carnivora, Rodentia, and Primata (38).

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