THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 23, Issue of June 8, pp. 20309–20315, 2001

Printed in U.S.A.

Tat1, a Novel Sulfate Transporter Specifically Expressed in Human Male Germ Cells and Potentially Linked to RhoGTPase Signaling*

Received for publication, December 27, 2000, and in revised form, February 27, 2001
Published, JBC Papers in Press, March 5, 2001, DOI 10.1074/jbc.M011740200

Aminata Touré‡§, Laurence Morin‡, Charles Pineau¶, Frédéric Becq‖, Olivier Dorseuil‡, and Gérard Gacon‡**

From the ‡Institut Cochin de Génétique Moléculaire, Département de Génétique, Développement et Pathologie Moléculaire, INSERM Unité 257, 24 Rue du Faubourg Saint-Jacques, 75014 Paris, France, the ¶GERM-INSERM Unité 435, Université de Rennes 1, Campus Beaulieu, Avenue du Général Leclerc, 35042 Rennes, France, and the ‖Laboratoire de Physiologie des Régulations Cellulaires, UMR 6558, Université de Poitiers, 40 Avenue du Recteur Pineau, 86022 Poitiers, France

RhoGTPases (Rho, Rac, and Cdc42) are known to regulate multiple functions, including cell motility, adhesion, and proliferation; however, the signaling pathways underlying these pleiotropic effects are far from fully understood. We have recently described a new RhoGAP (GTPase activating protein for RhoGTPases) gene, *MgcRacGAP*, primarily expressed in male germ cells, at the spermatocyte stage. We report here the isolation, through two-hybrid cloning, of a new partner of MgcRacGAP, very specifically expressed in the male germ line and showing structural features of anion transporters. This large protein (970 amino acids and a predicted size of 109 kDa), we provisionally designated Tat1 (for testis anion transporter 1), is closely related to a sulfate permease family comprising three proteins in human (DRA, Pendrin, and DTD); it is predicted to be an integral membrane protein with 14 transmembrane helices and intracytoplasmic NH_2 and COOH termini. *In situ* hybridization studies demonstrate that *Tat1* and *MgcRacGAP* genes are coexpressed in male germ cells at the spermatocyte stage. On testis sections, Tat1 protein can be immunodetected in spermatocytes and spermatids associated with plasma membrane. Two-hybrid and *in vitro* binding assays demonstrate that MgcRacGAP stably interacts through its NH_2-terminal domain with the Tat1 COOH-terminal region. Expression of Tat1 protein in COS7 cells generates a 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene and chloride-sensitive sulfate transport. Therefore we conclude that Tat1 is a novel sulfate transporter specifically expressed in spermatocytes and spermatids and interacts with MgcRacGAP in these cells. These observations raise the possibility of a new regulatory pathway linking sulfate transport to Rho signaling in male germ cells.

RhoGTPases, which include Rho, Rac, and Cdc42, are signaling proteins of the Ras superfamily that have been highly evolutionarily conserved from yeast to mammals. Members of the Rho family were initially believed to be primarily involved in the regulation of actin cytoskeleton and to control shape changes in response to extracellular growth factors. In recent years, they have been shown, in fibroblasts and other somatic cell types, to regulate a myriad of other cellular functions, including motility, adhesion, gene expression, cell cycle progression, and cell division (1). Although many potential effectors and regulatory proteins of Rho GTPases have been described, signaling pathways controlling their pleiotropic functions are far from fully understood (1–4).

We have recently described a new RhoGAP (GTPase activating protein for RhoGTPases) gene we called *Mgc(Male germ cell)RacGAP*, as it is primarily expressed in male germ cells, at spermatocyte stage (5). *MgcRacGAP* mRNA was also found in lower amount in many tissues in human and highly expressed in embryonic cerebral cortex in mouse (6). MgcRacGAP belongs to the chimaerin family of RhoGAPs, *i.e.* comprising a RhoGAP domain in the COOH-terminal half, and NH_2-terminal to this domain, a zinc finger-like motif and a large NH_2-terminal extension. We have recently described a mouse *MgcRacGAP* cDNA encoding an additional NH_2-terminal sequence of 106 residues resulting in a ~70-kDa protein (6); a human *MgcRacGAP* cDNA showing a homologous 5′ extension has also been reported (7, 8). Physiological functions of Chimaerins have been analyzed in details by microinjection of fibroblasts and neuroblastoma cells with various forms of *n*-chimaerin, a brain-specific Rac/Cdc42 GAP[1] (9). Whereas the GAP domain alone inhibited Rac-activated lamellipodia formation, strikingly, full-length *n*-chimaerin stimulated formation of the actin-based structures lamellipodia and filopodia. These *n*-chimaerin-stimulated events required the non-GAP NH_2-terminal half of the protein and were also dependent on activated Rac and Cdc42(10). These data strongly suggest that *n*-chimaerin mediates downstream signaling rather than down-regulation of Rac and Cdc42. Therefore, while the RhoGAP domain of MgcRacGAP exhibits GAP activity *in vitro* toward Rac and Cdc42 (5), we postulated that MgcRacGAP may also fulfil RhoGTPase effector functions through its non-GAP regions. A close homolog of MgcRacGAP is the *Drosophila* protein Rotund(Rn)RacGAP, the product of a spermatocyte-specific gene essential for male fertility, as its inactivation leads to male sterility in the fruit fly (11, 12). Altogether, these data

* This work was supported by INSERM and by grants from the Ligue Nationale Contre le Cancer and the Association pour la Recherche sur le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of fellowships from the Ministère de l'Éducation Nationale, de la Recherche et de la Technologie and from the Fondation pour la Recherche Médicale.

** To whom correspondence should be addressed: Institut Cochin de Génétique Moléculaire, Dépt. de Génétique, Développement et Pathologie Moléculaire, INSERM Unité 257, 24 Rue du Faubourg Saint-Jacques, 75014 Paris, France. Tel.: 33-1-44-41-24-70; Fax: 33-1-44-41-24-62; E-mail: gacon@cochin.inserm.fr.

[1] The abbreviations used are: GAP, GTPase activating protein; GST, glutathione *S*-transferase; aa, amino acid(s); FCS, fetal calf serum; BADG, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene.

indicate that MgcRacGAP may operate in a Rho-dependent signaling pathway(s) involved in the development and/or function of male germ cells.

To elucidate pathways implicating MgcRacGAP and approach their function, we have searched for partners of the MgcRacGAP NH_2-terminal region, expressed in male germ cells. We report here the cloning and characterization of an MgcRacGAP interacting protein specifically expressed in spermatocytes and spermatids and showing structural and functional features of a sulfate transporter.

EXPERIMENTAL PROCEDURES

Constructs—cDNAs encoding various domains of MgcRacGAP and Tat1 proteins were subcloned in pGEX plasmids for GST fusion protein production, in the pLex plasmid pVJL10 (13) for two-hybrid assays, and in the expression plasmid pRK5 for transient transfection experiments. Lex-Mgc(N), Lex-Mgc(GAP), and Lex-MgcRacGAP correspond to LexA fused to aa 1–180, aa 238–513 of MgcRacGAP, and full-length (aa 1–528) MgcRacGAP, respectively (amino acid numbering refers to MgcRacGAP sequence initially described in Ref. 5); GST-Mgc(N), GST-Mgc(GAP), and GST-MgcRacGAP correspond to the same MgcRacGAP regions fused to GST. GST-Tat1(Cter) corresponds to Tat1 (aa 664–970) fused to GST. pRK5-Tat1 and pRK5myctag-RacL61 express full-length Tat1 and myctagged-RacL61, respectively.

cDNA Cloning—Using Lex-Mgc(N) as a bait, we have screened a pACT2 human testis cDNA library (CLONTECH). Two hybrid procedures for isolation of specific clones that interact with MgcRacGAP were carried out according to published methods (13, 14). Full-length Tat1 cDNA was obtained by screening of λgt11 human testis cDNA libraries (CLONTECH).

Sequence Analysis—Clones of interest were sequenced using the Sanger dideoxy termination method adapted to an ABI 373A automated sequencer. Sequence analysis was performed using computer facilities provided by Infobiogen. BLAST programs were used to search for homologies in protein data banks, and the Transmembrane Protein secondary structure prediction program (TMpred) was used to elaborate a model of Tat1 topology across plasma membrane.

Northern Blot Analysis—We probed a multiple human tissue Northern blot (CLONTECH) with a *Tat1* cDNA fragment; a β-actin probe was used as a control.

In Situ Hybridization Experiments—A 1129-base pair fragment of *Tat1* cDNA was subcloned in the Bluescript KS plasmid allowing both SP6 and T7 transcription; ^{35}S-UTP-labeled antisense or sense cRNA probes were obtained by linearizing the plasmid, respectively, by *Hin*dIII or *Bam*HI and transcribing with T7 or SP6 RNA polymerase. In the same way, the *MgcRacGAP* cRNA antisense probe was generated by linearizing a pExLox plasmid containing a 1837-base pair cDNA fragment with *Kpn*I and transcribing with SP6 polymerase as described previously (5). 4-μm paraffin sections of human adult testis were hybridized overnight at 55 °C, washed as described previously (15), and exposed to Ilford K5 emulsion for 4 or 5 days. After development of the emulsion, sections were counterstained with toluidine blue and analyzed.

Cell Isolation—Sertoli cells were isolated from 20 days post-partum Harlan Sprague-Dawley rats as described previously (16). On the second day of culture, the cells were exposed to a hypotonic treatment to eliminate the contaminating germ cells (17). The degree of purity of the isolated Sertoli cells was greater than 98%. The isolation of peritubular cells was carried out during Sertoli cell preparation as described previously (18). The peritubular cells obtained were at least 99% pure. Spermatogonia were prepared from testes of 9 days post-partum male Harlan Sprague-Dawley rats. Seminiferous epithelial cells were dispersed by enzyme treatment and separated by sedimentation at unit gravity as described previously (19), leading to greater than 90% pure spermatogonia. Pachytene spermatocytes and early spermatids were prepared by centrifugal elutriation with a purity greater than 90% according to a method previously described (20), with the exception that enzymatic dissociation of cells was replaced by a mechanical dispersion. Immediately after isolation, all cell fractions were snap-frozen in liquid nitrogen and stored at −80 °C until used.

Antibodies—Antibodies to recombinant Tat1 COOH-terminal region (aa 664–970) were raised in rabbit and affinity-purified on a Tat1(aa 664–970)/Affi-Gel column.

Immunolocalization of Tat1 Protein—Testes of adult rats were carefully dissected out and immersed in Bouin's fixative solution for 24 h. Samples were then dehydrated through an increasing gradient of alcohol and acetone, before being embedded in paraffin wax. Testis sections (5 μm thick) were microwaved (2 × 3 min in phosphate-buffered saline) after mounting. Sections were then incubated with purified rabbit anti-Tat1 antibody, with a working dilution of 1:500. Preimmune serum was also used as a negative control. Complexes were revealed using a goat anti-rabbit biotinylated antibody (Dako, Glostrup, France) at a working dilution of 1:500, coupled with a streptavidin-peroxidase amplification combination. Sections were counterstained with hematoxylin. After color development, the samples were mounted with Glycergel (Dako, Glostrup, France) for microscopic observation.

Analysis of Tat1 Protein by Transient Transfection in HeLa and COS7 Cells—Transient transfections (24 or 48 h) of pRK5-*Tat1* and pRK5-*myctagged-racL61* expression vectors were performed in COS7 cells, using the fugene-6 reagent (Roche Molecular Biochemicals). Cells were washed in phosphate-buffered saline and then lysed in 50 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100. Lysates were loaded on a 10% SDS-acrylamide gel and electroblotted onto nitrocellulose. Rabbit anti-Tat1-purified antibodies were used to detect Tat1 expression in transfected cells.

For glycosylation inhibition experiments, transient transfection of HeLa cells was performed in a medium containing 10% FCS or 10% FCS supplemented with *N*- or *O*-glycosylation inhibitors: 25 ng/ml tunicamycin or 2 nM BADG (benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside), respectively. After 24 h, cells were lysed as above, and lysates were submitted to Western blot analysis.

In Vitro Transcription/Translation of Tat1—1 μg of Bluescript plasmids containing either *Tat1* full-length cDNA or *luciferase* cDNA were transcribed and translated in the presence of 20 μCi of [^{35}S]methionine in a rabbit reticulocyte lysate (Promega).

In Vitro Binding Assay—GST-Mgc(N), GST-Mgc(GAP), and GST-MgcRacGAP fusion proteins (1–3 μg) were coupled to 40 μl of glutathione-Sepharose beads and incubated with 2.5 μg of Tat1 COOH-terminal fragment (aa 664–970) during 1 h 30 min at 4 °C, in 50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100. Beads were sedimented and washed twice with cold washing buffer (25 mM Hepes, 125 mM NaCl, 1% Triton X-100, 0.1% SDS, protease inhibitors), once with 1 M NaCl, and once more with cold washing buffer. "Bound proteins" were recovered by boiling beads in Laemmli sample buffer and were subjected to a 12% SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose. Filters were incubated with rabbit anti-Tat1 antibodies followed by peroxidase-labeled swine anti-rabbit antibodies and developed using the ECL detection system (Amersham Pharmacia Biotech).

Transport Studies—COS7 cells were transiently transfected as described above with pRK5 empty vector (mock) or pRK5-expressing Tat1. Transport activity was assayed by measuring the efflux of sulfate ($^{35}SO_4$) as described (21, 22) or iodide (^{125}I) as described previously (23). All experiments were performed at 37 °C in 12-well plates. Cells were loaded with the respective radiolabeled anions (PerkinElmer Life Sciences, Boston, MA) for 30 min in a medium containing: 140 mM NaCl, 5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.4. For the sulfate experiment, the medium also contained [^{35}S]Na_2SO_4 (40 μCi/ml) and 0.3 mM Na_2SO_4. For iodide efflux determination, cells were loaded with $Na^{125}I$ (1 μCi/ml) in the presence of 1 μM KI. After incubation, cells were rinsed three times with cold medium. Efflux was determined every 1 min for 11 min during which the medium was removed at 1-min intervals to be counted and quickly replaced by 1 ml of the same medium. For the inhibition assay, 1 mM DIDS (4,4′-diisothiocyano-2,2′-disulfonic acid stilbene from Sigma) was added to the efflux buffer. For selectivity study, NaCl was substituted in the efflux buffer by sodium gluconate. At the end of the efflux, the medium was recovered, and cells were solubilized in 1 ml of 1 N NaOH. All fractions were counted for radioactivity, and efflux curves were constructed by plotting the percentage of total radioactivity released in the medium *versus* time. Results are expressed as means ± S.E. of n observations. To compare sets of data, we used either an analysis of variance or Student's t test. Differences were considered statistically significant when $p < 0.05$. All tests were performed using Prism 3.0 (Graphpad).

RESULTS AND DISCUSSION

In a search for partners of MgcRacGAP in male germ cells, we screened a two-hybrid library of human testis using as a bait a non-Rho-binding NH_2-terminal region (aa 1–180) of human MgcRacGAP sequence (5). Among others, a 1.3-kilobase cDNA was isolated; probing human RNA blots with this fragment revealed a 3.4-kilobase mRNA exclusively expressed in testis (Fig. 1). This 1.3-kilobase cDNA fragment was subse-

quently used as a probe to clone the full-length cDNA in human testis cDNA phage libraries. Analysis and sequencing of overlapping clones predicted an open reading frame of 2910 base pairs encoding a protein of 970 amino acids (109 kDa) of which only the COOH-terminal 300-aa fragment was present in the two-hybrid clone. Searching nucleic sequence data banks with this sequence allowed us to retrieve several human expressed sequence tag sequences showing a perfect match with our cDNA. Interestingly, all expressed sequence tag sequences found in data banks originate from testis, male germ cell tumors, or infant brain libraries, consistent with our finding that among adult human tissues expression of this gene is restricted to testis. Mouse and rat sequences encoding very similar proteins are also present in data banks, suggesting that orthologous genes exist in rodents. A human genomic fragment encompassing the whole cDNA sequence was also retrieved in the genomic section (High Throughput Genomic Sequence) of GenBank™. Blast search in protein data banks revealed a clear similarity of the predicted protein with a family of anion (sulfate) permeases comprising three members in human, *i.e.* DRA (24, 25), Pendrin (26), and DTD (27), and the protein was provisionally designated Tat1, for testis anion transporter 1; Tat1 appears most closely related to Pendrin (26% identity; 47% similarity) and DRA (28% identity; 46% similarity), which exhibit transport capacity for chloride/iodide and sulfate/chloride, respectively (26, 28). Multialignment programs (Fig. 2) demonstrate the presence of conserved blocks among the three proteins and Tat1-specific extra sequences in amino acids 600–652 and COOH-terminal regions; these additional sequences account for the longer size ot Tat1 (970 aa) as compared with DRA (764 aa) and Pendrin (780 aa). Sequence analysis also predicted several *N*-glycosylation sites in Tat1 sequence; in

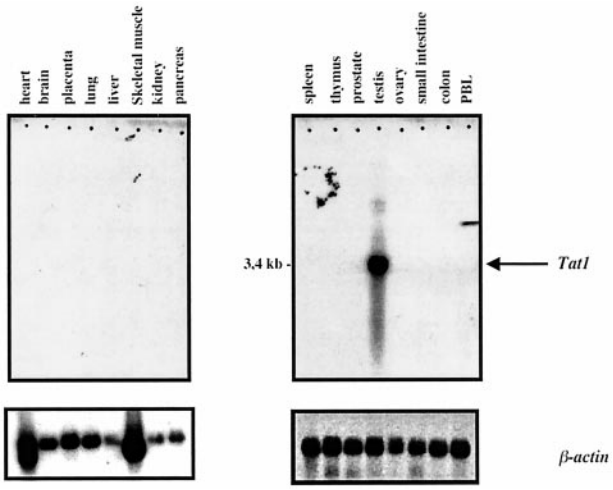

FIG. 1. **Tissue distribution of *Tat1* mRNA.** *Tat1* and *β−actin* cDNAs were used to probe multiple human tissue Northern blots (CLONTECH). Each lane contains 2 μg of poly(A)$^+$ RNA from the indicated tissues.

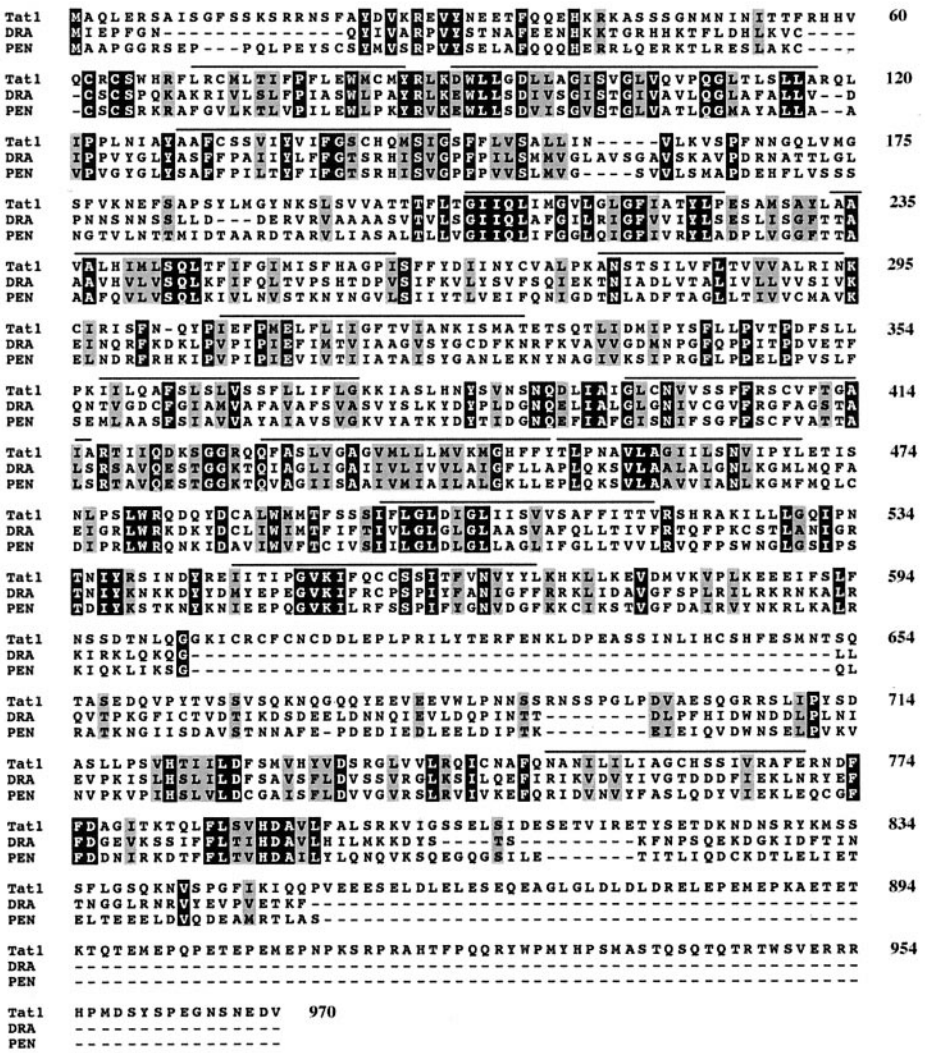

FIG. 2. **Tat1 protein sequence comparison.** Alignment of the full-length proteins Tat1, DRA, and Pendrin. Identities are *shaded black*, similarities are *shaded gray*, and predicted transmembrane domains in Tat1 are indicated by *dark lines*.

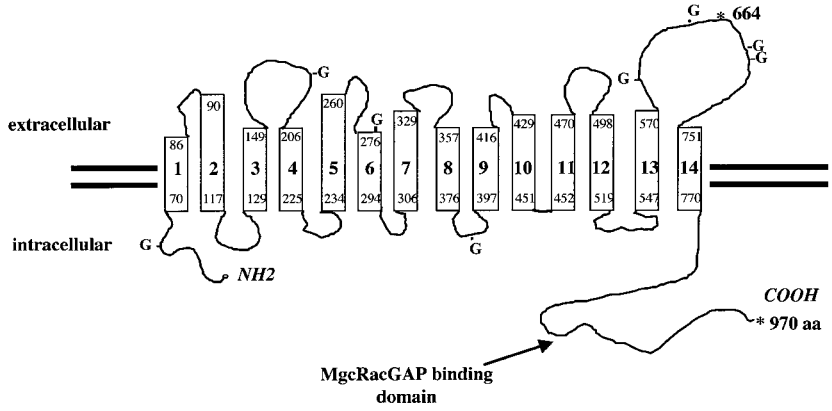

FIG. 3. **Proposed model for Tat1 protein.** Secondary structure prediction was performed using the "TMpred" program. *N*-Linked glycosylation sites are indicated by *G*. *Asterisks* demarcate the region of the Tat1 sequence (aa 664–970) encoded by the initial two-hybrid clone.

particular, there are four putative *N*-glycosylation sites in the major predicted external loop (amino acids 570–751) (see Fig. 3).

As expected from previous studies of DRA and Pendrin (26, 29), the hydrophobicity plot of the Tat1 protein exhibited multiple potential transmembrane helices, and membrane topology prediction programs (TMpred) led to a preferred model with 14 strong transmembrane regions and NH_2 and COOH termini inside the cytoplasm (Fig. 3).

Since *Tat1* gene appears exclusively expressed in testis on Northern blot (see Fig. 1), we looked for cell-specific expression in this organ. *In situ* hybridization of a *Tat1* cRNA probe to sections of human adult testis showed that *Tat1* expression is (i) detected only in a few tubule sections and (ii) restricted to germ cells, as the grain density over somatic cells was not detectably above background (Fig. 4*B*). Moreover, *Tat1* transcript was found primarily, if not exclusively, in spermatocytes. This pattern is similar to that previously observed for *MgcRacGAP* except for a low level expression of *MgcRacGAP* in round spermatids (5); consistent with this finding, *in situ* hybridization of two serial sections with *Tat1* and *MgcRacGAP* cRNA probes showed coexpression of the two genes within the same tubules in the same groups of germinal cells at the spermatocyte stage (Fig. 4). However, while all *Tat1*-positive tubule sections were found *MgcRacGAP*-positive as well, *MgcRacGAP* labeling was clearly detected in several *Tat1*-negative tubule sections, suggesting a shorter expression period for the *Tat1* gene.

Using affinity-purified antibodies directed to the COOH-terminal region of Tat1 protein (aa 664–970), we did not detect Tat1-specific signals on Western blots of whole human or rat testis extracts; this could reflect the facts that (i) the amount of Tat1 protein present in expressing cells may be quite low, as frequently observed for membranous ion transporters, and (ii) membranous proteins are usually very difficult to solubilize from whole testis extracts.[2] Western blot analysis on enriched populations of germ cells isolated from rat testis revealed multiple bands, ranging from 60 to 160 kDa and mainly expressed in spermatocytes and spermatids (Fig. 5); preincubation of antibodies with pure recombinant Tat1-COOH-terminal fragment resulted in the disappearance of the 60–160 kDa bands, demonstrating that this pattern corresponds to Tat1-related proteins (not shown). The complexity of the pattern of Tat1-related polypeptides probably results from glycosylation and proteolysis as discussed below. Immunostaining of rat testis sections using the same antibody also revealed specific stage-dependent labeling in seminiferous tubules (Fig. 6*A*). In the tubules, a strong labeling was observed in germ cells from spermatocytes to elongated spermatids (Fig. 6*B*). Immunoreactivity is clearly localized at the cell periphery of young spermatocytes, pachytene spermatocytes, and early and elongating spermatids, strongly suggesting a membranous expression of Tat1 (Fig. 6, *A* and *B*). A more diffuse cytoplasmic immunolabeling is still present in elongated spermatids at the very last steps of spermiogenesis (Fig. 6*B*). Altogether, data from Northern blot, *in situ* hybridization, and immunodetection experiments clearly demonstrate that the *Tat1* gene and Tat1 protein are exclusively expressed in spermatocytes and spermatids.

To further characterize Tat1 protein, we expressed *Tat1* cDNA in cultured cells. Transfection of *Tat1* constructs in

[2] A. Touré, L. Morin, C. Pineau, and G. Gacon, unpublished observation.

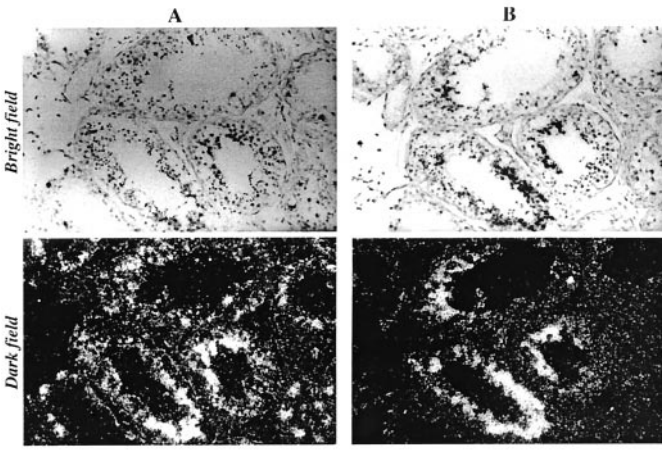

FIG. 4. ***In situ* hybridization analysis of *Tat1* and *MgcRacGAP* expression in human adult testis.** Two serial sections of human adult testis were hybridized with *MgcRacGAP* (*A*) and *Tat1* (*B*) cRNA probes, respectively. Pictures in bright field and dark field show the same three tubules on each section; mRNAs of the two genes are coexpressed in germ cells at the spermatocyte stage.

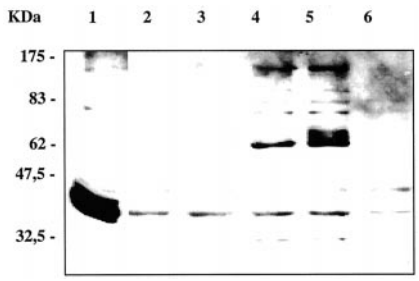

FIG. 5. **Western blot analysis of Tat1 in rat testis cell populations.** Enriched cell populations of rat testis were obtained as described under "Experimental Procedures"; detergent extracts from these cells were resolved by polyacrylamide gel electrophoresis, blotted onto nitrocellulose filters, and probed for Tat1 with an affinity-purified rabbit polyclonal antibody to the Tat1-COOH-terminal region (aa 664–970). *Lane 1*, 1 ng of recombinant Tat1 COOH-terminal region; *lane 2*, peritubular cells; *lane 3*, spermatogonia; *lane 4*, spermatocytes; *lane 5*, spermatids; *lane 6*, Sertoli cells.

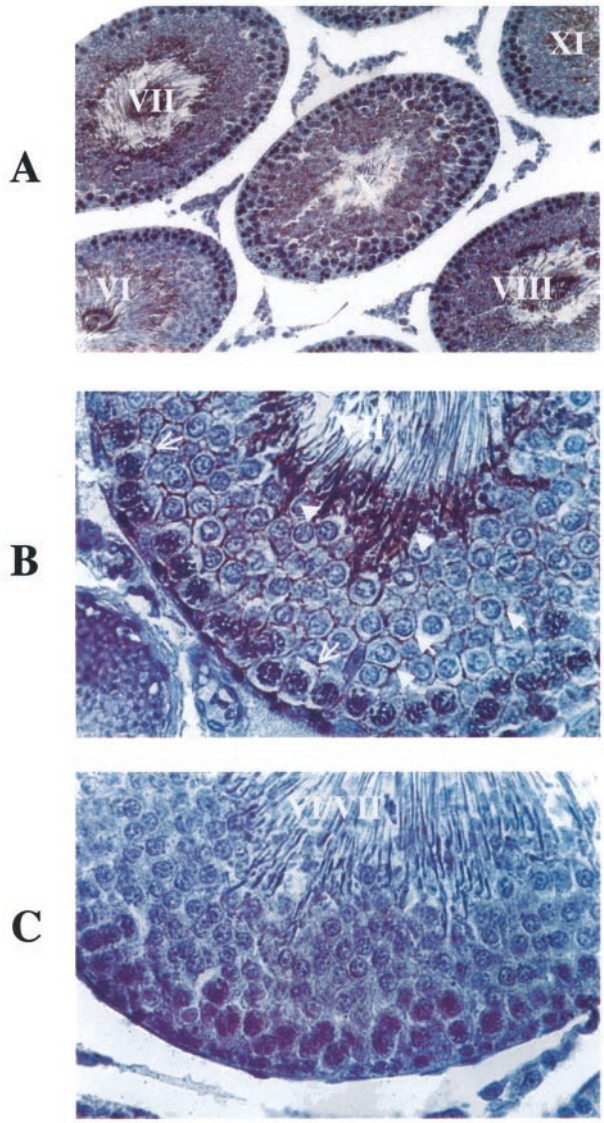

FIG. 6. **Immunodetection of Tat1 protein in adult rat testis.** Immunolocalization of Tat1 on rat testis sections was performed using rabbit polyclonal antibody to the Tat1-COOH-terminal region as a primary antibody, followed by a goat anti-rabbit biotinylated antibody and a streptavidin-peroxidase complex amplification combination. Sections were counterstained with Masson hematoxylin. *Roman numerals* indicate the stages of the seminiferous epithelium. *A*, stage-dependent expression of Tat1 protein. A specific stage-dependent labeling is distributed within the seminiferous epithelium. *B*, membranous localization of Tat1 protein. Tat1 immunoreactivity is detected at stage VII of the seminiferous epithelium in pachytene spermatocytes (*open arrows*) and early spermatids (*arrows*) at the periphery of the cytoplasm, suggesting a membranous localization. Elongated spermatids also display a positive but more diffuse cytoplasmic staining (*arrowheads*). *C*, preimmune control. The specificity of the immunoreaction was ascertained by a parallel experiment in which preimmune serum was used.

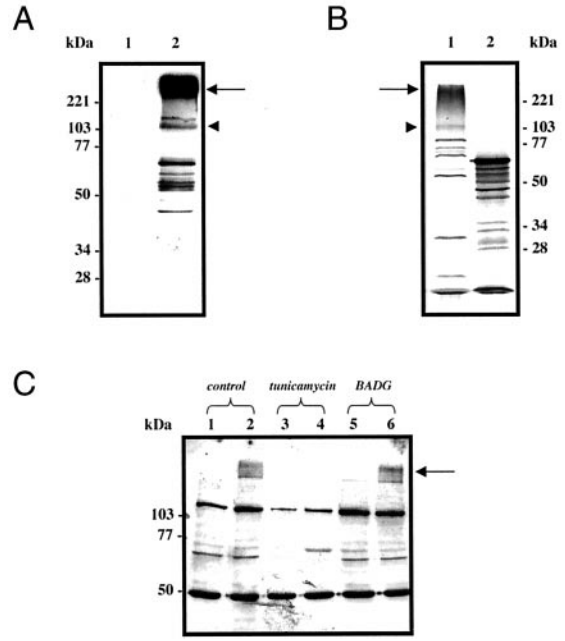

FIG. 7. **Analysis of Tat1 protein by transient transfection and *in vitro* transcription/translation assay.** *A*, detection of Tat1 protein in COS7 transfected cells. Immunodetection of Tat1 protein was performed in lysates from COS7 cells transiently transfected with *Tat1* (*lane 2*) and *racL61* (*lane 1*) expression vectors. *B*, *in vitro* transcription/translation of *Tat1* (*lane 1*) and control *Luciferase* (*lane 2*) cDNAs. In *A* and *B* *arrows* indicate high molecular mass bands corresponding to glycosylated forms, while *arrowheads* show putative unglycosylated full-length forms. *C*, inhibition of Tat1 glycosylation in HeLa cells. Transient transfection (24 h) of *Tat1* (*lanes 2*, *4*, and *6*) or *racL61* (*lanes 1*, *3*, and *5*) expression vectors was performed in HeLa cells, in medium containing either 10% FCS (*lanes 1* and *2*) or 10% FCS supplemented with glycosylation inhibitors: 25 ng/ml tunicamycin (*lanes 3* and *4*) and 2 nM BADG (*lanes 5* and *6*). Lysates were analyzed by immunoblotting.

COS7 (or HeLa cells) resulted in a high level of protein expression as evidenced by the strong specific immunofluorescence labeling of transfected cells (not shown). Immunoblots of lysates from COS7 cells overexpressing Tat1 protein show a heavily labeled diffuse band with a high molecular mass (>200 kDa) and a series of minor bands ranging from 110 to 50 kDa molecular mass (Fig. 7*A*). These bands are completely absent in a lysate from COS7 cells transfected with an irrelevant expression plasmid (pRK5-*racL61*) and thus must correspond to specific *Tat1* expression. This pattern is reminiscent of the one reported for the intestine-specific anion transporter DRA (29, 30) and suggests that the major slow migrating form of Tat1 could result from glycosylation of the protein, as usually found in integral membrane proteins. The 110-kDa band fits with the expected size of the protein as deduced from open reading frame translation (109 kDa) and might represent a minor fraction of non post-translationnally modified protein. The exact nature of the additional multiple sized proteins detected in COS7 lysates is not known; as mentioned previously for DRA, this complexity could result from a combination of NH_2-terminal truncation and glycosylation (30). *In vitro* transcription/translation experiments using *Tat1* cDNA as a template also resulted in a complex pattern of protein synthesized (Fig. 7B); this includes high molecular mass possibly glycosylated forms, 110-kDa and shorter bands, which could correspond to the use of multiple initiation sites.

Treatment of Tat1-expressing HeLa cells with tunicamycin, an inhibitor of *N*-glycosylation, resulted in the complete disappearance of the diffuse high molecular mass species (Fig. 7*C*); by contrast, *O*-glycosylation inhibitor BADG had no effects. This result clearly demonstrates that the bulk of Tat1 protein present in those cells is *N*-glycosylated. Altogether our data strongly suggest that within the different systems analyzed (HeLa human cells, COS7 simian cells, rat germ cells, and rabbit reticulocyte lysate) Tat1 protein is synthesized as a high molecular mass species corresponding to various degrees of glycosylation; this probably reflects species and/or tissue specificity of glycosyltransferase (31) and glycosidase (32) involved in *N*-glycan biosynthesis.

Tat1/MgcRacGAP interaction was analyzed in two-hybrid experiments and *in vitro* binding assays using recombinant proteins and anti-Tat1C-ter antibodies. In the yeast two-hybrid assay, Tat1 COOH-terminal region strongly interacts with the

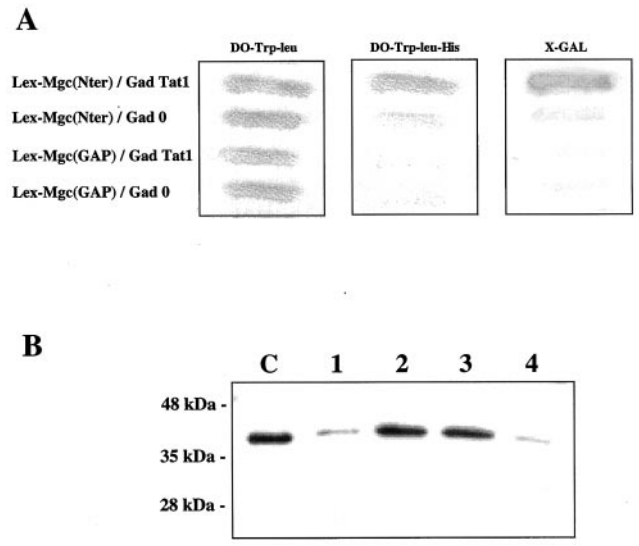

FIG. 8. **Analysis of Tat1/MgcRacGAP interaction.** *A*, two-hybrid experiments. L40 yeasts were cotransformed with Gad plasmids expressing either GAL_4-Tat1 or GAL_4 alone and Lex plasmids encoding either Lex-Mgc(N) or Lex-Mgc(GAP). Transformants were plated on nonselective medium (*DO-Trp-Leu*), selective medium (*DO-Trp-Leu-His*), and subjected to a β-galactosidase assay (*X-GAL*). *B*, *in vitro* binding assay. Equal amounts of Tat1-(Cter) protein (aa 664–970) were incubated with glutathione-Sepharose beads either linked to GST alone or GST fused to different domains of MgcRacGAP: *lane 1*, GST; *lane 2*, GST-Mgc(N); *lane 3*, GST-MgcRacGAP; *lane 4*, GST-Mgc(GAP). After incubation at 4 °C during 1 h 30 min, beads were washed and boiled in SDS sample buffer. Fractions were analyzed by immunoblot using anti-Tat1C-ter antibodies. (2 ng of Tat1-Cter protein were also loaded on the gel for control (*C*).)

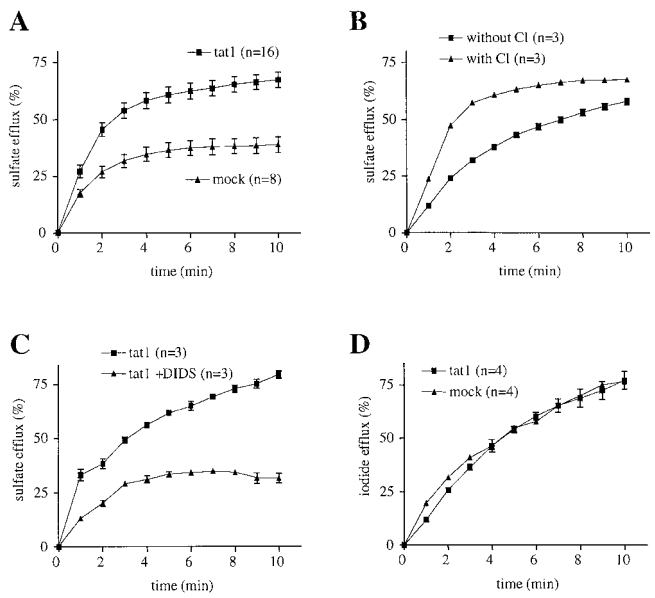

FIG. 9. **Sulfate and iodide transport in COS7 cells expressing Tat1.** The sulfate transport is expressed as a percentage of efflux as a function of time. Results are expressed as means ± S.E. of *n* observations as indicated for each experimental condition. *A*, detection of sulfate transport in Tat1-expressing COS7 cells compared with mock transfected cells. *B*, effect of the gluconate replacement (noted without Cl^-) of chloride (noted with Cl^-) on the sulfate efflux in Tat1-expressing cells. *C*, inhibition of sulfate efflux by 1 mM DIDS in cells expressing Tat1. *D*, iodide efflux in mock and Tat1-expressing cells.

Mgc-N region (aa 1–180) but not with the GAP domain (Fig. 8*A*). *In vitro*, GST-Mgc-N fusion protein actually binds to the carboxyl-terminal domain of Tat1. The Tat1 COOH-terminal region also interacts with the full-length MgcRacGAP protein, but not with the GAP domain (Fig. 8*B*, *lanes 2–4*). Taken together, these results demonstrate that MgcRacGAP interacts through its NH_2-terminal region with the cytoplasmic COOH-terminal domain of Tat1.

In a first approach to Tat1 function, COS7 cells expressing Tat1 were assayed for anion transport. As shown in Fig. 9*A*, sulfate efflux was markedly enhanced in Tat1-expressing cells as compared with mock transfected cells. When extracellular chloride was replaced by gluconate, the Tat1-mediated sulfate transport was markedly reduced showing a dependence upon extracellular Cl^- (Fig. 9*B*). In addition, Tat1-mediated sulfate efflux was inhibited by the anion exchanger inhibitor DIDS as illustrated in Fig. 9*C*. By contrast, the iodide transport (Fig. 9*D*) was not modified by Tat1 expression. Together, these data support the idea that Tat1 is a *bona fide* anion transporter displaying sulfate transport at least partly through a sulfate/chloride exchange mechanism (21, 22).

As mentioned earlier (see Figs. 2 and 3), the Tat1 COOH-terminal region has no counterpart in DRA, Pendrin, and DTD and is predicted to be located in cytoplasm; this suggests that MgcRacGAP binding to the Tat1 COOH-terminal region could provide a specific regulatory mechanism of Tat1 transport function and that Rho GTPases might participate in the regulation of sulfate transport in male germ cells.

Although we have no clue as to the role of sulfate transport in male germ cells, two lines of evidence support the hypothesis that the MgcRacGAP/Tat1 pathway could fulfil important functions. 1) Recently, the orthologous gene of *MgcRacGAP* in *Caenorhabditis elegans*, *CYK-4*, and human *MgcRacGAP* have been shown to play an essential role in the process of cell division (8, 33). More specifically, those proteins have been shown to associate with mitotic spindle and to be required for cytokinesis. Interestingly, throughout spermatogenesis, both mitotic and meiotic cell divisions are characterized by incomplete cytokinesis resulting in the persistence of intercytoplasmic bridges (34). While incomplete cytokinesis during mitotic divisions may be related to the absence of MgcRacGAP in spermatogonia (5),[2] we speculate that, in spermatocytes, where MgcRacGAP and Tat1 are coexpressed, Tat1/MgcRacGAP interaction may interfere with MgcRacGAP function and eventually preclude complete cytokinesis during meiotic divisions.

2)Tat1 belongs to a family of anion transporters comprising three human proteins, *i.e.* DRA, DTD, and Pendrin. Strikingly, mutations in all three corresponding genes *Dra*, *DTD,* and *PDS* are responsible for human diseases, *i.e.* congenital chloride diarrhea (28), diastrophic dysplasia (27, 35, 36), and goiter associated with congenital deafness known as Pendred syndrome (26, 37), respectively. This implies that each of these genes plays a specific and essential role in affected tissues. In agreement with this view, *Dra* and *PDS* show very specific expression in intestine epithelium and thyroid, respectively. As *Tat1* gene expression appears restricted to the male germ line at the spermatocyte stage, we speculate that Tat1 may fulfil critical functions in these cells and that mutations in the *Tat1* gene may result in impaired spermatogenesis in human.

Acknowledgments—We are grateful to F. Letourneur, N. Lebrun, and C. Jougla for excellent technical assistance. We thank Drs. P. Camparo and M. Arborio for providing human testis sections and P. Chaffey for advice in protein analysis.

REFERENCES

1. Van Aelst, L., and D'Souza-Schorey, C. (1997) *Genes Dev.* **11,** 2295–2322
2. Hall, A. (1998) *Science* **279,** 509–514
3. Aspenstrom, P. (1999) *Curr. Opin. Cell Biol.* **11,** 95–102
4. Marshall, C. (1999) *Curr. Opin. Cell Biol.* **11,** 732–736
5. Touré, A., Dorseuil, O., Morin, L., Timmons, P., Jégou, B., Reibel, L., and Gacon, G. (1998) *J. Biol. Chem.* **273,** 6019–6023
6. Arar, C., Ott, M. O., Touré, A., and Gacon, G. (1999) *Biochem. J.* **343,** 225–230
7. Kawashima, T., Hirose, K., Satoh, T., Kaneko, A., Ikeda, Y., Kaziro, Y.,

Nosaka, T., and Kitamura, T. (2000) *Blood* **96,** 2116–2124
8. Hirose, K., Kawashima, T., Iwamoto, I., Nosaka, T., and Kitamura, T. (2001) *J. Biol. Chem.* **276,** 5821–5828
9. Ahmed, S., Kozma, R., Monfries, C., Hall, C., Lim, H. H., Smith, P., and Lim, L. (1990) *Biochem. J.* **272,** 767–773
10. Kozma, R., Ahmed, S., Best, A., and Lim, L. (1996) *Mol. Cell. Biol.* **16,** 5069–5080
11. Agnel, M., Roder, L., Vola, C., and Griffin-Shea, R. (1992) *Mol. Cell. Biol.* **12,** 5111–5122
12. Guichard, A., Bergeret, E., and Griffin-Shea, R. (1997) *Mech. Dev.* **61,** 49–62
13. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) *Cell* **74,** 205–214
14. Kaiser, C., Michaelis, S., and Mitchell, A. (1989) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Wilkinson, D. G., and Green, J. (1990) in *Post-implantation Mammalian Embryos: A Practical Approach* (Copp, A. L., and Cockroft, D. L., eds) pp. 55–72, IRL Press, Oxford
16. Toebosch, A. M., Robertson, D. M., Klaij, I. A., de Jong, F. H., and Grootegoed, J. A. (1989) *J. Endocrinol.* **122,** 757–762
17. Galdieri, M., Ziparo, E., Palombi, F., Russo, M. A., and Stefanini, M. (1981) *J. Androl.* **5,** 249–259
18. Skinner, M. K., and Fritz, I. B. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 114–118
19. Guillaume E. D. A., Moertz E., Courtens, J. L., Jégou, B., and Pineau, C. (2001) *Proteome* 2000 in press
20. Pineau, C., Syed, V., Bardin, C. W., Jégou, B., and Cheng, C. Y. (1993) *J. Androl.* **14,** 87–98
21. Elgavish, A., and Meezan, E. (1992) *Am. J. Physiol.* **263,** C176—C186
22. Satoh, H., Susaki, M., Shukunami, C., Iyama, K., Negoro, T., and Hiraki, Y. (1998) *J. Biol. Chem.* **273,** 12307–12315
23. Becq, F., Hamon, Y., Bajetto, A., Gola, M., Verrier, B., and Chimini, G. (1997) *J. Biol. Chem.* **272,** 2695–2699
24. Schweinfest, C. W., Henderson, K. W., Suster, S., Kondoh, N., and Papas, T. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4166–4170
25. Hoglund, P., Haila, S., Scherer, S. W., Tsui, L. C., Green, E. D., Weissenbach, J., Holmberg, C., de la Chapelle, A., and Kere, J. (1996) *Genome Res.* **6,** 202–210
26. Scott, D. A., Wang, R., Kreman, T. M., Sheffield, V. C., and Karnishki, L. P. (1999) *Nat. Genet.* **21,** 440–443
27. Hastbacka, J., de la Chapelle, A., Mahtani, M. M., Clines, G., Reeve-Daly, M. P., Daly, M., Hamilton, B. A., Kusumi, K., Trivedi, B., Weaver, A., Coloma, A., Lovett, M., Buckler, A., Kaitila, I., and Lander, E. S. (1994) *Cell* **78,** 1073–1087
28. Moseley, R. H., Hoglund, P., Wu, G. D., Silberg, D. G., Haila, S., de la Chapelle, A., Holmberg, C., and Kere, J. (1999) *Am. J. Physiol.* **276,** G185—G192
29. Byeon, M. K., Westerman, M. A., Maroulakou, I. G., Henderson, K. W., Suster, S., Zhang, X. K., Papas, T. S., Vesely, J., Willingham, M. C., Green, J. E., and Schweinfest, C. W. (1996) *Oncogene* **12,** 387–396
30. Byeon, M. K., Frankel, A., Papas, T. S., Henderson, K. W., and Schweinfest, C. W. (1998) *Protein Expression Purif.* **12,** 67–74
31. Dennis, J. W., Granovsky, M., and Warren, C. E. (1999) *Bioessays* **21,** 412–421
32. Herscovics, A. (1999) *Biochim. Biophys. Acta* **1473,** 96–107
33. Jantsch-Plunger, V., Gonczy, P., Romano, A., Schnabel, H., Hamill, D., Schnabel, R., Hyman, A. A., and Glotzer, M. (2000) *J. Cell Biol.* **149,** 1391–1404
34. Weber, J. E., and Russell, L. D. (1987) *Am. J. Anat.* **180,** 1–24
35. Hastbacka, J., Kerrebrock, A., Mokkala, K., Clines, G., Lovett, M., Kaitila, I., de la Chapelle, A., and Lander, E. S. (1999) *Eur. J. Hum. Genet.* **7,** 664–670
36. Superti-Furga, A., Rossi, A., Steinmann, B., and Gitzelmann, R. (1996) *Am. J. Med. Genet.* **63,** 144–147
37. Coyle, B., Reardon, W., Herbrick, J. A., Tsui, L. C., Gausden, E., Lee, J., Coffey, R., Grueters, A., Grossman, A., Phelps, P. D., Luxon, L., Kendall-Taylor, P., Scherer, S. W., and Trembath, R. C. (1998) *Hum. Mol. Genet.* **7,** 1105–1112