Functional equivalence of human X- and Y-encoded isoforms of ribosomal protein S4 consistent with a role in Turner syndrome

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Several genes are found on both the human X and Y chromosomes in regions that do not recombine during male meiosis. In each case, nucleotide sequence analysis suggests that these X–Y gene pairs encode similar but nonidentical proteins. Here we show that the human Y- and X-encoded ribosomal proteins, RPS4Y and RPS4X, are interchangeable and provide an essential function: either protein rescued a mutant hamster cell line that was otherwise incapable of growth at modestly elevated temperatures. These findings are consistent with the hypothesis that RPS4 deficiency has a role in Turner syndrome, a complex human phenotype associated with monosomy X.

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Molecular findings are causing geneticists to reconsider the previously-held notion[1] that the mammalian Y chromosome is virtually devoid of genes. Interestingly, eight of the nine genes cloned from the human or mouse Y chromosome have homologues on the X chromosome. Some X–Y gene pairs[2–5] are located in the pseudoautosomal region, where X–Y recombination enforces identity. Other X–Y genes[6–10] are located in strictly sex-linked regions, where the X and Y chromosomes do not recombine. Apart from the sex-determining gene, *SRY*, three genes have been cloned from the strictly sex-linked portion of the human Y chromosome: *ZFY* (zinc finger protein), *RPS4Y* (ribosomal protein S4) and *AMGY* (amelogenin). All three have counterparts on the X chromosome (*ZFX*, *RPS4X* and *AMGX*), and in each case the products of the X and Y genes appear, based on nucleotide sequence analysis, to be similar but distinct[6–8].

Though the proteins encoded by such X- and Y-linked genes are not identical, they might be functionally equivalent. Such equivalence would make X inactivation of a gene unnecessary, since XX and XY embryos would have the same dosage of that gene. In this context, it is noteworthy that *ZFX* and *RPS4X* escape X inactivation[6,7]. Nonetheless, in no case has functional equivalence of X- and Y-encoded isoforms — or even the Y-encoded protein's existence — been demonstrated.

These issues are particularly important with respect to *RPS4Y*, which together with its X homologue, *RPS4X*, has been hypothesized to play a critical role in Turner syndrome (see below). Human *RPS4X* encodes a 263-amino acid protein identical in sequence to rat ribosomal protein S4, a component of the 40S subunit[11]. Like most ribosomal proteins, S4 is highly conserved: the rat and human proteins are identical to their homologues in mouse[12] and hamster (see below), differ from the chicken homologue by only four amino acid substitutions (unpublished results), and are 73% identical to the yeast homologue (which is essential for growth)[13]. In yeast, two distinct genes encode identical S4 proteins. By contrast, the human Y homologue, *RPS4Y*, encodes a protein that differs from RPS4X by 19 amino acid substitutions[7]. As no isoforms of any other mammalian ribosomal protein are known, this degree of divergence calls into question RPS4Y's ability to substitute for RPS4X. We addressed this issue by complementation analysis of a temperature sensitive mutant, tsBN63.

A temperature-sensitive *RPS4* mutation

The tsBN63 cell line was obtained by mutagenizing BHK21/13 male (XY) hamster cells with nitrosoguanidine[14]. When tsBN63 cells are shifted from 33.5 °C to 39.5 °C, protein synthesis decreases rapidly and cell division ceases[15]. Somatic cell hybrid experiments have demonstrated that tsBN63's temperature sensitivity is recessive[14]. A human gene complementing the tsBN63 mutation was recently cloned by DNA-mediated gene transfer and found to encode RPS4X (ref. 15).

To identify the tsBN63 mutation, we first cloned and sequenced *Rps4* cDNAs from BHK21/13, the wild-type cell line. The cDNAs were found to encode a protein identical in sequence to human RPS4X. We then cloned the *Rps4* coding sequence from tsBN63 by reverse transcription-polymerase chain reaction amplification. The tsBN63 coding sequence differed from wild type by a single nucleotide substitution, a guanine to adenine transition, corresponding to a glycine to arginine

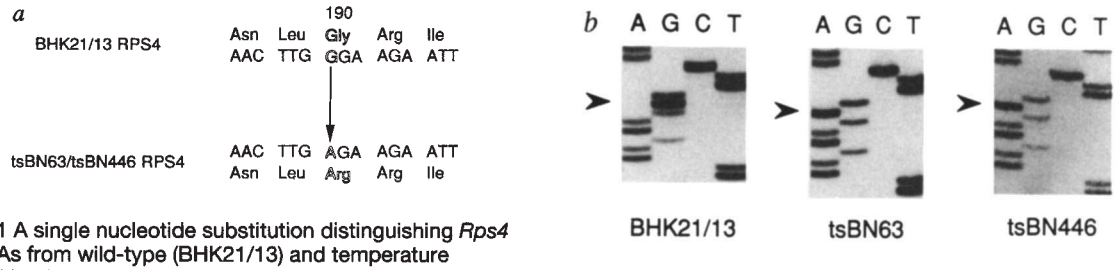

Fig. 1 A single nucleotide substitution distinguishing *Rps4* cDNAs from wild-type (BHK21/13) and temperature sensitive (tsBN63 and tsBN446) hamster cell lines. *a*, Wild-type and mutant nucleotide and amino acid sequences near the codon 190. *b*, Autoradiographs of nucleotide sequencing around the mutation site. Sequences are shown 5' to 3' from top to bottom. Arrowhead indicates site of G to A substitution.

substitution in the protein (Fig. 1). Surprisingly, the same mutation was found in tsBN446, an independently isolated temperature sensitive mutant of BHK21/13 belonging to the tsBN63 complementation group[16]. This recurrent mutation may be at a hot spot for nitrosoguanidine-induced transitions. (Like mouse[17,18], hamster probably has a single S4-encoding gene[15], X-linked and present in only one copy in XY cells.)

To confirm that this *Rps4* mutation caused the temperature sensitivity, we tested the ability of wild type or mutant hamster *Rps4* cDNAs to restore growth of tsBN63 cells at 39.5 °C (Table 1). Prior to transfection, cDNA sequences were inserted into a vector containing a hygromycin B resistance (Hb^r) gene[19]. Transfection efficiencies of wild-type (BHK21/13) and mutant (tsBN63) hamster cDNAs were similar, as shown by the number of Hb^r colonies that appeared at 33.5 °C. When cells were shifted to 39.5 °C soon after electroporation, only transfection with wild type *Rps4* cDNA yielded many Hb^r colonies. Simultaneous selection for Hb^r and growth at 39.5 °C, as compared with Hb selection alone, dramatically reduced the number of colonies, as observed previously[15]. In another experiment (rightmost column in Table 1), temperature sensitivity was tested only after adaptation to Hb. In this case, most Hb^r colonies arising from transfection with wild-type cDNA were also temperature resistant (the reason some colonies failed to grow at 39.5 °C is not known), unlike colonies transfected with mutant cDNA. This experiment confirmed that the plasmid bearing the wild-type *Rps4* cDNA conferred resistance to both Hb and temperature. We concluded that tsBN63's temperature sensitivity is due to the single amino acid change in RPS4.

Table 1 Wild-type hamster and human cDNA clones transform tsBN63 to temperature resistance

cDNA clone transfected	Number of colonies		
	33.5 °C + Hb	39.5 °C + Hb	39.5 °C/33.5 °C[a]
BHK21/13 *Rps4*	204	17	6/10
tsBN63 *Rps4*	217	1[b]	0/10
Human *RPS4X*	231	49	9/10
Human *RPS4Y*	253	29	7/10
Vector alone	220	0	0/10[b]

[a]For each transfection, ten Hb^r colonies that had appeared at 33.5 °C were replicaplated and incubated at 39.5 °C or 33.5 °C in medium containing Hb. The number of colonies that regrew at each temperature is shown.
[b]A revertant (spontaneous frequency 3.3×10^{-8}; ref. 14).

Human *RPS4Y* complements the tsBN63 mutation

We took advantage of tsBN63's temperature sensitivity to test whether the human RPS4Y protein can substitute for RPS4X. *RPS4X* or *RPS4Y* cDNAs were inserted into the Hb^r vector and transfected into tsBN63 cells. These cells were then cultured in the presence of Hb at either 33.5 °C or 39.5 °C (Table 1). Transfection efficiencies were similar with the two plasmids, as judged by the number of Hb^r colonies that appeared at 33.5 °C. At 39.5 °C, transfections with either human *RPS4X* or *RPS4Y* yielded many Hb^r colonies. Again, simultaneous selection for Hb^r and growth at 39.5 °C yielded fewer colonies than Hb^r selection alone. The experiment was repeated three times with similar results (not shown). When temperature sensitivity was assayed after adaptation to Hb (rightmost column in Table 1), most *RPS4X* and *RPS4Y* transfectants isolated at 33.5 °C also grew at 39.5 °C, as was observed with transfectants that had received the wild-type hamster

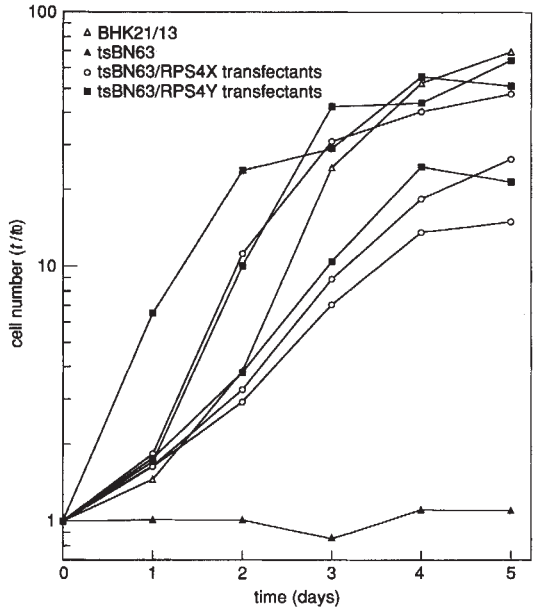

Fig. 2 Growth curves of BHK21/13, tsBN63, and several independent temperature resistant transfectants at 39.5 °C. Cells were plated onto 35 mm dishes (approximately 10^4 cells per dish) and incubated at 33.5 °C for 24 h. At that time (t_o), the cells were placed in fresh medium and shifted to 39.5 °C. Every 24 h thereafter, cells of one dish were trypsinized and counted using a Coulter counter. The ordinate gives the ratio of cell number at time t to cell number at t_o. Expression of the plasmid in each transfectant was confirmed by RT–PCR (not shown).

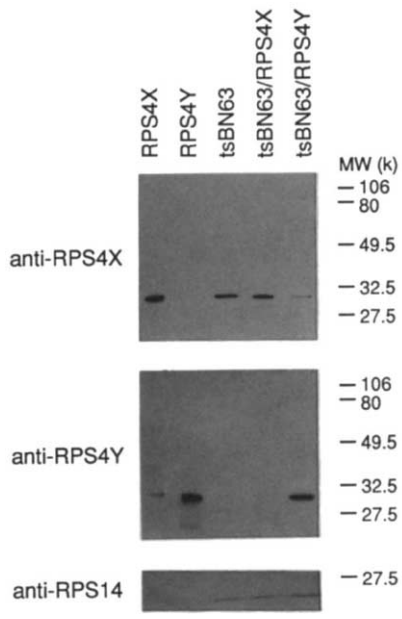

Fig. 3 Western blot analysis of S4 protein in ribosomes of tsBN63 cells and temperature resistant transfectants. Protein extracts from the following sources were probed sequentially with RPS4Y, RPS14 and RPS4X antisera: *E. coli* (140 ng) expressing *RPS4X* or *RPS4Y*; ribosomes (10 μg) from tsBN63 cells growing at 33.5 °C or from *RPS4X* or *RPS4Y* transfectants growing at 39.5 °C. The RPS14 protein (M_r 16,000) migrated with the dye front.

Rps4 cDNA. Moreover, at 39.5 °C, the growth curves of *RPS4X* and *RPS4Y* transfectants were similar to that of wild type cells (Fig. 2). Their rates of protein synthesis at 39.5 °C were also similar (not shown). Thus, as judged by a variety of assays, both human *RPS4X* and *RPS4Y* complemented tsBN63's *Rps4* mutation.

RPS4Y replaces hamster RPS4 in ribosomes

To investigate the nature of the complementation, we tested whether the RPS4Y protein was present in ribosomes of a temperature-resistant *RPS4Y* transfectant. Anti-peptide sera that discriminate between RPS4X and RPS4Y were used for Western blotting (Fig. 3). The RPS4X antiserum also detected the hamster tsBN63 RPS4 protein, since the epitope it recognized was not altered by the tsBN63 mutation. As expected, only hamster RPS4 or human RPS4X was detected in ribosomes purified from the *RPS4X* transfectant. In contrast, human RPS4Y was detected in ribosomes of the *RPS4Y* transfectant, and the amount of hamster RPS4 protein, as detected by the RPS4X antiserum, was greatly reduced. A control antiserum detected similar amounts of RPS14, another 40S subunit protein, in tsBN63 and the two transfectants. Since RPS14 and RPS4 are stoichiometric components of ribosomes, the amount of RPS4 (hamster or human) must also have been similar in the three samples. Thus, in ribosomes of tsBN63 cells rescued by *RPS4Y*, the mutant hamster RPS4 protein was almost totally replaced by human RPS4Y.

We conclude that human RPS4X and RPS4Y function interchangeably in ribosomes, despite their 19 amino acid differences. Apart from post-translational modifications, no other isoforms of mammalian ribosomal proteins are known. Functionally equivalent isoforms of other ribosomal proteins exist in yeast, although these differ by no more than a few amino acids[20]. While the present data suggest that ribosomes containing RPS4X or RPS4Y function identically, we cannot exclude the possibility that in certain circumstances RPS4Y-containing ribosomes, present only in males, might serve a distinct role; neither can we exclude the possibility that RPS4Y or RPS4X might have a second function apart from the ribosome.

Discussion

RPS4X and *RPS4Y* are the first X–Y homologous genes shown to encode nonidentical but functionally interchangeable proteins. This interchangeability meshes well with the observation that the human *RPS4X* gene escapes X inactivation: the existence of a Y-linked equivalent obviates dosage compensation of an X-linked gene. Perhaps other X-Y gene pairs (for example, *ZFX/ZFY* or mouse *Ube1x/Ube1y*) will also be found to encode interchangeable isoforms. In any case, the deep-seated notion that the sex-linked portion of the Y chromosome functions only in male differentiation[1] may have to be modified to encompass aspects of development and physiology that occur in both sexes. Indeed, we have shown here that the strictly sex-linked portion of the human Y chromosome can provide a function required for cell viability.

Our findings may be relevant to Turner syndrome, a human phenotype associated with monosomy X (ref. 21). Embryos with a 45,X karyotype develop as females with poor viability *in utero*, and those that survive exhibit ovarian failure, short stature and specific somatic abnormalities that may include lymphedema, webbing of the neck and aortic coarctation[22]. These phenotypes may result from haploidy for genes that are common to the X and Y chromosomes and that escape X inactivation[23]; different phenotypic components may be due to different genes. Attempts to localize such "Turner genes" on the X chromosome by karyotype/phenotype correlation have proved difficult, in part because of mosaicism frequently associated with X chromosome anomalies and because observers often did not report which phenotypic components were present or absent. However, efforts to localize a Y-linked Turner gene are providing a more coherent understanding: XY females carrying deleted Y chromosomes can be divided into those with and without somatic Turner features. XY females with such features consistently lack a 90-kb region located between *SRY* and *ZFY* (ref. 24; D.C.P., manuscript in preparation). (Apart from a role in stature[25], the pseudoautosomal region appears not to contribute to the Turner phenotype; ref. 26; D.C.P., manuscript in preparation.) Exploration of this 90-kb region resulted in identification of one gene, *RPS4Y*, which satisfies the prediction for a Turner gene: an X-linked homologue escapes X inactivation. It was postulated that normal human development requires at least two *RPS4* genes per cell (for example, two *RPS4X* genes, as in XX females, or one *RPS4X* and one *RPS4Y* gene, as in XY males); and that the Turner phenotype is due, at least in part, to the presence of just one *RPS4* gene[7]. This model predicted that the RPS4X and RPS4Y proteins are functionally equivalent, as demonstrated here. Just *et al.* have recently suggested[27] that *RPS4* is not a Turner

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gene, because cell lines from some Turner patients with structurally abnormal X chromosomes have two or more active copies of *RPS4X*. However, the authors did not report which aspects of the Turner phenotype their patients displayed (XY females with *RPS4Y* deletions show a specific phenotypic subset; D.C.P., manuscript in preparation), nor did they address the problem of 45,X mosaicism, making it difficult to assess their findings. Our present studies demonstrate that *RPS4Y* and *RPS4X* share an essential function. Whether a quantitative defect in that function contributes to the Turner phenotype is not yet certain.

Methodology

Hamster Rps4 cDNA cloning and sequencing. Hamster wild-type *Rps4* cDNAs were isolated from a λgt10 library by hybridization with a human *RPS4X* cDNA. Two independent cDNAs were subcloned and sequenced (GenBank, EMBL, and DDBJ accession number D11087). For temperature sensitive mutants tsBN63 and tsBN446, cDNA was reverse transcribed from total RNA with the *Rps4* primer 5'-AGCACAGTTGCCTAGAAGGGC-3'. The *Rps4* coding sequence was then PCR amplified using this primer and a second primer, 5'-TTCGCGGCCGCCGCAGCCATG-3'. Thermocycling conditions were 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C for 25 cycles in buffer recommended by Perkin Elmer-Cetus. Products were subcloned into pUC118 and sequenced. Three clones from tsBN63 and four clones from tsBN446 all had the same nucleotide sequence.

Transfections. Hamster or human *RPS4* cDNAs subcloned into the pcDEB vector[19] were transfected into tsBN63 cells as described[15]. Approximately 10^6 transfected cells were plated onto a 100-mm dish, incubated for two days at 33.5 °C, and then incubated at either 33.5 °C or 39.5 °C following addition of 300 μg ml^{-1} Hb. After two weeks, colonies were fixed, stained and counted[14]. In mock transfections omitting DNA, no Hb^r colonies were observed.

Preparation of ribosomal proteins and immunoblotting. Human RPS4X and RPS4Y proteins were expressed in *E. coli* using the pET–3d vector[28]. To purify mammalian ribosomes, frozen pellets of 5×10^6 cells were homogenized in 1 ml of buffer containing 1% Triton X–100, 0.2 M sucrose, 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 20 mM Tris, pH 7.5 and centrifuged 5 min at 15,000*g*. Post-mitochondrial supernatants were centrifuged for 20 min at 436,000*g* in a Beckman TL100.2 rotor. Pellets were resuspended in homogenization buffer containing 1% deoxycholate instead of Triton X–100 and ribosomes were pelleted through 10% sucrose cushions by centrifugation for 6 h at 200,000*g* in a Beckman SW41 rotor. Ribosomal proteins were extracted in Laemmli buffer, fractionated on 12% SDS–PAGE gels and transferred in 25 mM AMPSO, pH 9.5 to nitrocelluose[29]. Rabbit antisera were raised against peptides corresponding to amino acids 155 through 174 of the RPS4X and RPS4Y proteins[7] and affinity purified using peptide columns. The RPS4X antiserum did not react with RPS4Y. The RPS4Y antiserum, after incubation for 2 h with 1 μg ml^{-1} of the RPS4X peptide, showed only minor reactivity with RPS4X. Immunodetection was with the Amersham chemiluminescence system. Filters were stripped by washing for 30 min at 50 °C in 2% sodium dodecyl sulphate, 100 mM 2–mercaptoethanol, 62.5 mM Tris pH 6.7 prior to reprobing.

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1. Ohno, S. *Sex chromosomes and sex-linked genes.* (Springer-Verlag, New York, 1967).
2. Goodfellow, P.J., Darling, S. M., Thomas, N. S. & Goodfellow, P.N. A pseudoautosomal gene in man. *Science* **234**, 740–743 (1986).
3. Gough, N. M. *et al.* Localization of the human GM-CSF receptor gene to the X-Y pseudoautosomal region. *Nature* **345**, 734–736 (1990).
4. Ellison, J. *et al.* Directed isolation of human genes that escape X inactivation. *Somat. Cell molec. Genet.* **18**, 259–268 (1992).
5. Schiebel, K., Weiss, B., Wöhrle, D. & Rappold, G. A human pseudoautosomal gene, ADP/ATP translocase, escapes X-inactivation whereas a homologue on Xq is subject to X-inactivation. *Nature Genet.* **3**, 82–87 (1993).
6. Schneider-Gädicke, A., Beer-Romero, P., Brown, L.G., Nussbaum, R. & Page, D.C. *ZFX* has a gene structure similar to *ZFY*, the putative human sex determinant, and escapes X inactivation. *Cell* **57**, 1247–1258 (1989).
7. Fisher, E.M.C. *et al.* Homologous ribosomal protein genes on the human X and Y chromosomes: Escape from X inactivation and possible implications for Turner syndrome. *Cell* **63**, 1205–1218 (1990).
8. Salido, E.C., Yen, P. H., Koprivnikar, K., Yu, L-C. & Shapiro, L.J. The human enamel protein gene amelogenin is expressed from both the X and the Y chromosomes. *Am. J. hum. Genet.* **50**, 303–316 (1992).
9. Mitchell, M.J., Woods, D.R., Tucker, P.K., Opp, J.S. & Bishop, C.E. Homology of a candidate spermatogenic gene from the mouse Y chromosome to the ubiquitin-activating enzyme E1. *Nature* **354**, 483–486 (1991).
10. Kay, G.F. *et al.* A candidate spermatogenesis gene on the mouse Y chromosome is homologous to ubiquitin-activating enzyme E1. *Nature* **354**, 486–489 (1991).
11. Wool, I.G., Chan, Y-L., Paz, V. & Olvera, J. The primary structure of rat ribosomal proteins: The amino acid sequences of L27a and L28 and corrections in the sequences of S4 and S12. *Biochim. Biophys. Acta* **1050**, 69–73 (1990).
12. Zinn, A. R. *et al.* Inactivation of the *Rps4* gene on the mouse X chromosome. *Genomics* **11**, 1097–1101 (1991).
13. Synetos, D., Dabeva, M. D. & Warner, J. R. The yeast ribosomal protein S7 and its genes. *J. biol. Chem.* **267**, 3008–3013 (1992).
14. Nishimoto, T. & Basilico, C. Analysis of a method for selecting temperature-sensitive mutants of BHK cells. *Somat. Cell Genet.* **4**, 323–340 (1978).
15. Watanabe, M. *et al.* Molecular cloning of the human gene, *CCG2*, that complements the BHK-derived temperature-sensitive cell cycle mutant tsBN63—identity of *CCG2* with the human X-chromosomal *SCAR/RPS4X* gene. *J. cell Sci.* **100**, 35–43 (1991).
16. Nishimoto, T. *et al.* Large-scale selection and analysis of temperature-sensitive mutants for cell reproduction from BHK cells. *Somat. Cell Genet.* **8**, 811–824 (1982).
17. Ashworth, A., Rastan, S., Lovell-Badge, R. & Kay, G. X-chromosome inactivation may explain the difference in viability of XO humans and mice. *Nature* **351**, 406–408 (1991).
18. Hamvas, R. M. *et al. Rps4* maps near the inactivation center on the mouse X chromosome. *Genomics* **12**, 363–367 (1992).
19. Hayakawa, H., Koike, G. & Sekiguchi, M. Expression and cloning of complementary DNA for a human enzyme that repairs O^6-methylguanine in DNA. *J. molec. Biol.* **213**, 739–747 (1990).
20. Woolford, J. J. The structure and biogenesis of yeast ribosomes. *Adv. Genet.* **29**, 63–118 (1991).
21. Ford, C.E., Jones, K. W., Polani, P.E., De Almeida, J.C. & Briggs, J.H. A sex-chromosome anomaly in a case of gonadal dysgenesis (Turner's syndrome). *Lancet* **i**, 711–713 (1959).
22. Lippe, B. Turner syndrome. *Endocrinol. Metab. Clin. North Am.* **20**, 121–152 (1991).
23. Ferguson-Smith, M.A. Karyotype-phenotype correlations in gonadal dysgenesis and their bearing on the pathogenesis of malformations. *J. med. Genet.* **2**, 142–155 (1965).
24. Page, D.C., Fisher, E.M., McGillivray, B. & Brown, L.G. Additional deletion in sex-determining region of human Y chromosome resolves paradox of X,t(Y;22) female. *Nature* **346**, 279–281 (1990).
25. Ballabio, A. *et al.* Contiguous gene syndromes due to deletions in the distal short arm of the human X chromosome. *Proc. natn. Acad. Sci. U.S.A.* **86**, 10001–10005 (1989).
26. Levilliers, J., Quack, B., Weissenbach, J. & Petit, C. Exchange of terminal portions of X- and Y-chromosomal short arms in human XY females. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2296–2300 (1989).
27. Just, W., Geerkens, C., Held, K.R. & Vogel, W. Expression of RPS4X in fibroblasts from patients with structural aberrations of the X chromosome. *Hum. Genet.* **89**, 240–242 (1992).
28. Studier, F.W. & Moffatt, B.A. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. molec. Biol.* **189**, 113–130 (1986).
29. Szewczyk, B. & Kozloff, L.M. A method for the efficient blotting of strongly basic proteins from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose. *Anal. Biochem.* **150**, 403–407 (1985).