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Genetics

Genetics of the large, external, transformation-sensitive (LETS) protein: Assignment of a gene coding for expression of LETS to human chromosome 8

(fibronectin/external membrane proteins/cell hybrids/cancer)

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ABSTRACT **Techniques have been developed to analyze the genetics of the large, external, transformation-sensitive (LETS) protein (fibronectin). External membrane proteins of human–mouse somatic cell hybrids with reduced numbers of human but not mouse chromosomes were labeled by lactoperoxidase-catalyzed iodination. Cell surface proteins were identified after sodium dodecyl sulfate/polyacrylamide gel electrophoresis by autoradiography of the dried gel. The LETS protein was identified in parental human cells, and LETS segregated in human–mouse cell hybrids formed from human WI-38 fibroblasts and a mouse L-cell line not expressing LETS. The LETS protein segregated concordantly with the chromosome 8 enzyme marker glutathione reductase (EC 1.6.4.2) and human chromosome 8. These findings demonstrate that a gene, *LETS*, encoded on chromosome 8, is responsible for the LETS protein expression in humans. Because LETS has been implicated in tumorigenicity and cellular transformation, it is of interest that rearrangement or modifications in the number of chromosome 8 have been associated with certain forms of cancer.**

A glycoprotein of high molecular weight has been identified on the surface of a variety of fibroblast cells by using procedures that label cell surface proteins with radioactive compounds or specific antibodies (1–8). This protein has been named the large, external, transformation-sensitive (LETS) protein (fibronectin) and is often absent or greatly reduced in many transformed cells (2). The LETS protein has been suggested to have a role in cell adhesion, morphology, and surface architecture, and in decreased contact inhibition of movement and loss of microfilament bundles of many neoplastic cells (9–13). Absence of this protein from the surface of a variety of different cell lines generally correlated with tumorigenicity (14, 15). A decrease in LETS protein after transformation of chicken fibroblasts was shown to result from decreased biosynthesis, as well as increased turnover (16). The 5- to 6-fold decrease in biosynthesis is apparently due to decreased quantities of translatable LETS messenger RNA (17).

Little is known about the genetics of LETS expression. Human–mouse cell hybrids have proved important for genetically analyzing the human genome (18). Because human chromosomes are preferentially lost in human–rodent cell hybrids, a partial human chromosome complement offers the opportunity to determine linkage relationships and a framework for mapping genes responsible for the expression of the LETS protein. Evidence is reported for the linkage (synteny) of a gene coding for LETS protein expression to the chromosome 8 gene coding for glutathione reductase (GSR, EC 1.6.4.2) and the assignment of the gene encoding LETS expression to human chromosome 8.

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MATERIALS AND METHODS

Parental and Hybrid Cells. The parental cells were WI-38, a karyotypically normal human embryonic lung fibroblast [American Type Culture Collection (ATCC) CCL 75], and LTP, a heteroploid thymidine kinase (TK) and hypoxanthine phosphoribosyltransferase deficient cell derived from the LM/TK$^-$ mouse line (19). LM/TK$^-$ cells were used for iodination and enzyme marker analysis, being identical to LTP for cell surface proteins and enzyme markers analyzed in this study. WI-38 fibroblasts were grown on Eagle's basal medium (GIBCO) supplemented with 10% fetal calf serum, penicillin, streptomycin, and kanamycin (20). Mouse parental cells were propagated on Dulbecco's modified Eagle's medium (DMEM) (20). Parental cells were fused with β-propriolactone-inactivated Sendai virus in monolayer, and hybrid cells were cloned and maintained on hypoxanthine/aminopterin/thymidine (HAT) selection medium consisting of DMEM and HAT (19). These cell hybrids were isolated and characterized previously (19, 21).

Normal and Simian Virus 40 (SV40)-Transformed Human Fibroblasts. WI-38 (CCL 75) and SV40-transformed WI-38, WI-38 VA 13 subline 2RA (CCl 75.1), were obtained from the ATCC. Normal skin fibroblasts (GM-37), Lesch–Nyhan skin fibroblasts (GM-177), SV40-transformed GM-37 skin fibroblasts (GM-637), and SV40-transformed GM-177 Lesch–Nyhan skin fibroblasts (GM-847) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ).

Iodination. Parental and hybrid cells were iodinated by using a procedure based on that of Phillips and Morrison (22) as modified by Hubbard and Cohn (23). Confluent monolayers in 25-cm^2 Falcon flasks were washed three times with Hanks' balanced salt solution (HBSS). The labeling solution consisted of HBSS that was 0.1 M N-[tris(hydroxymethyl)methyl]glycine (Tricine), pH 7.6, and 7 mM in glucose. To this mixture (2.2 ml final volume) was added 60 milliunits of lactoperoxidase (Sigma) and 600 μCi (1 Ci = 3.7×10^{10} becquerels) of carrier-free ^{131}I (Amersham/Searle). The reaction was started by addition of 35 milliunits of glucose oxidase (Sigma). Iodination was conducted for 30 min at 25°C and stopped by washing three times with HBSS containing 1 mM phenylmethylsulfonyl fluoride (Sigma). Cells were then scraped with a rubber policeman into a solution of HBSS plus 1 mM phenylmethylsulfonyl fluoride. Specific activities of 10^6 cpm of trichloroacetic acid-insoluble material per mg of protein were routinely obtained.

Abbreviations: LETS protein, large external transformation-sensitive protein (fibronectin); GSR, glutathione reductase; TK, thymidine kinase; SV40, simian virus 40; HBSS, Hanks' balanced salt solution; NaDodSO$_4$, sodium dodecyl sulfate; EMP, external membrane protein.

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Trypsinization of Iodinated Cells. Iodinated cells were washed three times with HBSS and incubated in 2 ml of a solution of HBSS containing 20 μg of trypsin treated with tosylamido-2-phenylethyl chloromethyl ketone (259 units/mg, Worthington Biochemicals) for 15 min at 37°C. Cells were then scraped with a rubber policeman into a solution of HBSS containing 1 mM phenylmethylsulfonyl fluoride.

External Membrane Protein Electrophoresis. Sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide electrophoresis was performed as described by Laemmli (24). The gels were cast in 1.5-mm-thick slabs in a 10-cm electrophoresis apparatus from Hoeffer Scientific Instruments. Scraped cells were pelleted and dissolved in a solution containing 0.06 M Tris·HCl at pH 6.8, 2% $NaDodSO_4$, 10% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.001% bromphenol blue by heating at 100°C for 3 min. Ten to twenty μl containing approximately 100 μg of protein and 100,000 trichloroacetic acid-precipitable cpm of sample was loaded per gel slot, and electrophoresis was carried out at 30 mA per slab, constant current. Following electrophoresis, the gels were fixed and stained for protein with Coomassie brilliant blue R250 in 50% methanol/7% acetic acid (vol/vol). Molecular weight standards—chymotrypsinogen (25,000), ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase *a* (92,000), β-galactosidase (130,000), and myosin (210,000)—were run on the same gel as the iodinated proteins. Gels were destained overnight in 50% methanol/7% acetic acid. Gel slabs were prepared for autoradiography by first swelling the gel in 7% acetic acid and then drying the gel onto filter paper by heating under reduced pressure for 3 hr in a Hoeffer drying apparatus. ^{131}I was visualized by autoradiography on Kodak XR-2 x-ray film.

Enzyme Marker Electrophoresis. Cell homogenates from confluent monolayers were prepared in 0.05 M Tris·HCl, pH 7.5, at concentrations of 6.0–8.0 × 10^7 cells per ml (19, 21). Supernatant fractions of cell homogenates were examined by vertical gel electrophoresis (Buchler Instruments). Electrophoresis of GSR was accomplished in 12% starch gels (Electrostarch Co.) containing a 1:20 dilution of the stock buffer composed of 0.1 M Tris and 4.5 mM EDTA, titrated to a pH of 9.6 with 6 M NaOH. The electrode buffers consisted of the undiluted stock buffer (25). Electrophoresis was carried out at 230 V (constant voltage) for 20 hr at 4°C. GSR activity was observed by histochemical staining. A 15-ml solution consisting of 0.05 M Tris·HCl, pH 8.0, containing 3.7 mM oxidized glutathione (60 mg) (Sigma) and 0.7 mM TPNH (14 mg) (Sigma) was added to 10 ml of 0.8% (wt/vol) molten (50°C) agar and layered over the cut gel. GSR activity was visualized after 1 hr at 37°C (25). The gel electrophoresis procedures for the 24 other enzyme markers coded by genes assigned to each human chromosome have been previously reported (Table 1).

RESULTS

Identification of the LETS Protein in Human Fibroblasts. Cell surface proteins were identified by labeling the cell surface by lactoperoxidase-catalyzed iodination using ^{131}I followed by electrophoresis on a 6% $NaDodSO_4$/polyacrylamide gel (Fig. 1). Lactoperoxidase, a macromolecule of about 150,000 molecular weight, does not cross the lipid bilayer of intact cells, and therefore under appropriate conditions can catalyze the iodination of only externally disposed cell surface proteins (27, 28). Individual proteins were classified by molecular weight as determined with molecular weight standards by $NaDodSO_4$/polyacrylamide gel electrophoresis. The external membrane proteins (EMPs) of 195,000, 175,000, and 130,000 molecular weights were designated EMP-195, EMP-175, and EMP-130, respectively (Fig. 1) (26). The 250,000 molecular weight protein was designated LETS protein because it fit the generally accepted criteria (2) of high molecular weight, labeling by external labeling procedures, abundance in nontransformed fibroblasts such as diploid fibroblast WI-38, and absence or marked reduction in transformed cells such as the mouse heteroploid LM/TK^- parental line (Fig. 1). This protein is also extremely sensitive to trypsin treatment of the cell surface, and exponentially growing cells have lower levels on their surfaces than density-inhibited cells.

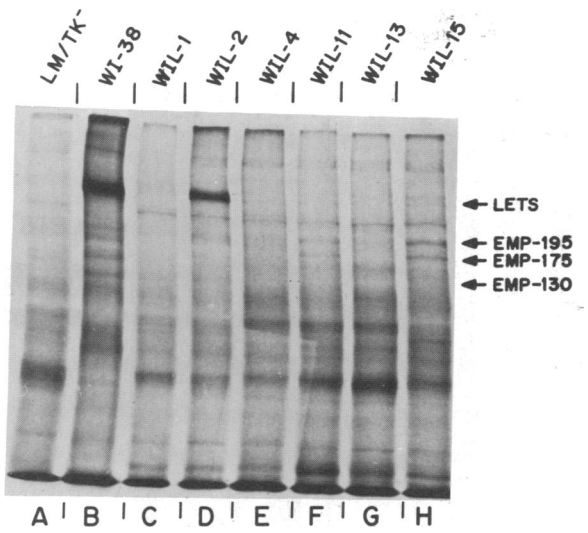

FIG. 1. Autoradiogram of ^{131}I-labeled cell surface proteins analyzed on a 6% $NaDodSO_4$/polyacrylamide gel. Protein bands represent external membrane proteins of mouse, human, and cell hybrids. Channel A, mouse line LM/TK^-; B, human diploid fibroblasts WI-38; C–H, human–mouse cell hybrids. LETS-positive hybrid WIL-2 in D and LETS-negative hybrids in C and E–H. EMP-130, 175, and 195 are human external membrane proteins that segregate independently of LETS in cell hybrids (26).

Expression of LETS in Human–Mouse Cell Hybrids. In some cell hybrids the LETS protein was expressed. For example, LETS was present in primary hybrids WIL-2 (Fig. 1, channel D; Fig. 2, channel 2) and WIL-8 (Fig. 2, channel 3) and secondary hybrids WIL-8R and WIL-8X (Fig. 2, channels 6 and 8). LETS in hybrid cells had the same molecular weight, sensitivity to trypsin, and density dependence as LETS in human fibroblasts. Absence or reduced expression of LETS in transformed cells was of interest because hybrid cells have transformed characteristics and express LETS (see *Discussion*). The human parent, WI-38, was investigated to determine if LETS could be expressed after transformation by SV40. WI-38 fibroblasts transformed by SV40, WI-38 VA 13 (ATCC CCL 75.1), were labeled by lactoperoxidase-catalyzed iodination, and the labeled polypeptides were separated on a 6% $NaDodSO_4$/polyacrylamide gel. These transformed cells do express abundant amounts of LETS protein. Furthermore, two additional human lines, normal skin fibroblasts (GM-37) and Lesch–Nyhan skin fibroblasts (GM-177), both continue to express LETS protein after SV40 transformation. This finding, that human LETS protein is still expressed in transformed fibroblasts, strongly suggests that the LETS protein appearing in transformed hybrid cells is of human origin (see Discussion).

Genetic Linkage of LETS and GSR. Syntenic relationships of the gene coding for the expression of LETS protein were investigated by examining the segregation patterns of LETS and human enzyme markers previously assigned to each of the human chromosomes. Syntenic relationships refer to genes

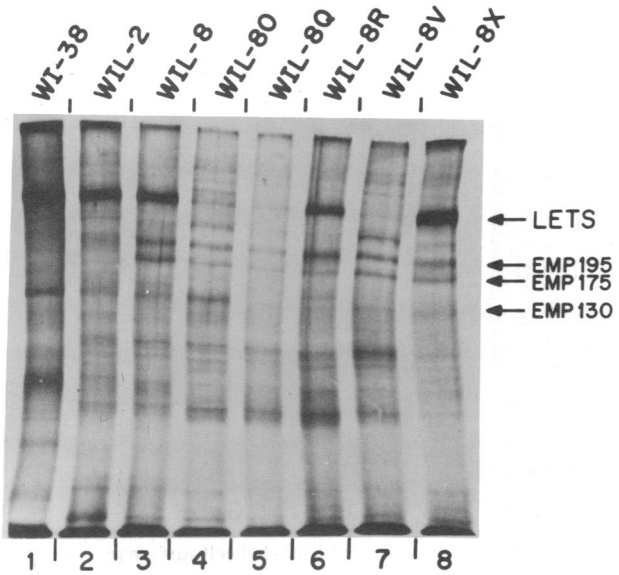

FIG. 2. External membrane proteins of human cells and cell hybrids. Autoradiogram of ^{131}I-labeled cell surface proteins analyzed on a 6% NaDodSO$_4$/polyacrylamide gel. Channel 1, human diploid fibroblast WI-38; 2–8 human–mouse cell hybrids. LETS is expressed in channels 1–3, 6, and 8.

coded on the same chromosome. Twenty-four primary cell hybrids were tested for the presence and absence of LETS and its cosegregation with 26 human enzyme markers (Table 1). These enzymes represent markers for all 22 human autosomes and the X chromosome. WI-38 fibroblasts are derived from female embryonic lung cells and therefore do not contain the Y chromosome (38). The LETS protein showed concordant segregation with human GSR, an enzyme marker encoded on human chromosome 8 (Fig. 3, Table 1), but discordant segregation with all other human enzymes tested (Table 1). Only 5.5% discordancy was observed between segregation of LETS and GSR in primary cell hybrids (which is best explained by either chromosome breakage or differential sensitivity of the assays for LETS and GSR). The syntenic relationship was further examined in 15 secondary clones isolated from primary hybrid WIL-8 for the segregation of LETS and GSR. Nine subclones retained both GSR and LETS, while five lost both and one lost LETS but retained GSR. All other enzyme markers segregated independently. The continued segregation of LETS and GSR in secondary clones suggests that the genes coding for GSR and LETS expression are on the same chromosome.

The asyntenic relationships between LETS and other enzyme markers representing all other human chromosomes in 50 primary and secondary cell hybrids combined excludes assignment of the gene required for the expression of the LETS protein to any of the other 23 human chromosomes. Synteny with *GSR*, previously assigned to chromosome 8, and these results, taken together, demonstrate that a gene required for the expression of the LETS protein is encoded on human chromosome 8. Because the mouse parental cell is deficient for TK and hypoxanthine phosphoribosyltransferase, cell hybrids must retain chromosomes 17 and X, respectively. To conclusively exclude these two chromosomes, a LETS- and GSR-positive hybrid was counterselected separately on 8-azaguanine and bromodeoxyuridine-supplemented media to eliminate chromosomes X and 17, respectively (cf. ref. 20). Hybrids without enzyme markers for chromosomes 17 and X continued to express LETS and GSR.

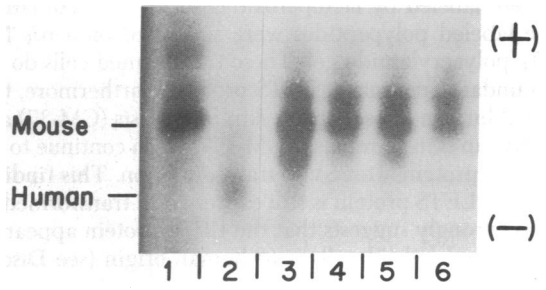

FIG. 3. GSR isozyme patterns of mouse, human, and cell hybrids. Channel 1, mouse; 2, human; 3–5, cell hybrid extracts of WIL-2, WIL-8, and WIL-10S are positive for human GSR activity; 6, WIL-15 is negative for human GSR activity. Intermediate activity between human and mouse parental bands supports a dimeric structure for glutathione reductase.

Table 1. Segregation of LETS with enzyme markers in primary human–mouse cell hybrids

Chromosome	Enzyme marker	LETS	
		Concordant	Discordant
1	PGM_1/PEPC	12	12
2	IDH_S/MDH_S	16	6
3	βGAL_A	10	5
4	PEPS	15	8
5	HEX_B	11	11
6	ME	14	5
7	GUS	15	6
8	*GSR*	*17*	*1*
9	AK_1	19	4
10	GOT_S	13	10
11	LDH_A	10	13
12	LDH_B/PEPB	16	8
13	ESD	17	7
14	NP	13	10
15	MPI	15	6
16	APRT	14	4
17	TK*	3	21
18	PEPA	15	9
19	GPI	18	6
20	ADA	13	11
21	SOD_S	8	16
22	$ACON_M$	6	9
X	G6PD	11	13

Chromosome assignments of genes coding for the enzyme markers have been summarized (29, 30, 31). The concordant segregation column shows the number of hybrid clones in which LETS and an enzyme marker(s) were either present or absent together. The discordant segregation column represents the expression of only the external membrane protein or an enzyme marker(s), but not both. The enzyme markers analyzed by gel electrophoresis were PGM_1 (phosphoglucomutase-1); PEPC (peptidase C); IDH_S (isocitrate dehydrogenase, soluble); MDH_S (malate dehydrogenase, soluble); HEX_B (hexosaminidase B); ME (malic enzyme); GUS (β-glucuronidase) (32); GOT_S (glutamate oxaloacetate transaminase, soluble); LDH_A (lactate dehydrogenase A); LDH_B (lactate dehydrogenase B); PEPB (peptidase B); NP (nucleoside phosphorylase); MPI (mannose-phosphate isomerase); APRT (adenine phosphoribosyltransferase); PEPA (peptidase A); GPI (glucose-phosphate isomerase); ADA (adenosine deaminase); SOD_S (superoxide dismutase, soluble); and G6PD (glucose-6-phosphate dehydrogenase). Additional enzyme markers were βGAL_A (β-galactosidase A) (33); PEPS (peptidase S) (34); GSR (25); AK_1 (adenylate kinase 1) (35); ESD (esterase D) (36); and $ACON_M$ (aconitase, mitochondrial) (37).

* TK has been assigned to human chromosome 17, which is required for growth in human–mouse (TK^-) hybrids maintained in hypoxanthine/aminopterin/thymidine selection medium.

Chromosome Assignment of LETS. Chromosome analysis of primary and secondary cell hybrids was performed to confirm the chromosome assignment made by segregation analysis. Concordant segregation was observed between LETS and

Table 2. Chromosome distribution in human–mouse cell hybrids segregating LETS and GSR

Hybrid	LETS	GSR	Chromosome 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X
WIL-2	+	+	−	−	−	−	−	−	−	+	−	+	−	+	−	−	+	−	+	−	−	−	+	−	+
WIL-8	+	+	+	−	−	+	+	−	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	−	+
WIL-8X	+	+	−	−	+	+	+	−	+	+	−	+	+	+	−	+	−	−	+	+	+	+	+	−	+
WIL-8Y	−	−	−	−	−	−	−	+	+	−	−	+	+	−	−	+	−	−	+	+	−	+	+	−	+
WIL-10S	+	+	−	+	+	−	+	−	−	+	−	−	+	−	−	+	−	−	+	+	−	+	+	+	+
WIL-11	−	−	−	−	+	+	+	+	−	−	−	+	+	−	+	+	+	−	+	+	−	+	+	+	+
WIL-15	−	−	−	−	−	+	−	−	+	−	−	+	+	+	−	+	−	−	+	+	−	−	+	+	+
WIL-19J	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	+	−	+	−	+	−	−	−	+	+

GSR, LETS, and human chromosomes were scored for their presence (+) and absence (−) in hybrid cells. Cell hybrids were prepared and scored for human chromosomes as reported previously (20, 35).

human chromosome 8 (Table 2). All other human chromosomes were excluded from assignment considerations because of discordant segregation. GSR and LETS segregated concordantly in these hybrids with human chromosome 8, thus confirming the assignment of *LETS* and *GSR* to chromosome 8.

DISCUSSION

The 250,000 molecular weight protein was identified as the LETS protein by the generally accepted criteria of high molecular weight, labeling by external labeling procedures, extreme sensitivity to proteases, density dependence, and absence or marked reduction on the surfaces of many transformed cells (2). Because the cell hybrids tested have typical transformed phenotypes, it was of interest that LETS protein was expressed in some human–mouse cell hybrids. There are examples of spontaneously transformed fibroblasts (2) and adenovirus-2-transformed rat embryo lines (14) that produce LETS protein. As reported here, human WI-38, normal skin fibroblasts, and Lesch–Nyhan skin fibroblasts also express LETS protein after SV40 transformation. However, the absence of LETS protein from the cell surface correlates better with tumorigenicity than with cell transformation (14, 15). In agreement with these findings, a number of SV40-transformed human lines, including SV40-transformed Lesch–Nyhan skin fibroblasts, are nontumorigenic when injected into *nude* mice (39).

Because human LETS and LETS from nontransformed mouse cells have identical molecular weights (40), there is a possibility, in the assay systems employed, that LETS in hybrid cells is due to the activation of mouse LETS protein, although our results support the hypothesis that LETS in the hybrid cells we tested is of human origin. This conclusion is based on the finding that LETS in transformed parental mouse LM/TK$^-$ cells is not expressed, yet human LETS is expressed in parental human cells and after SV40 transformation. It is reasoned, then, that LETS is of human origin in these human–mouse cell hybrids with characteristics of transformed cells. Additional evidence in support of a human origin for LETS in hybrid cells is that dependence on cellular density and trypsin sensitivity remained the same as for LETS in human fibroblasts.

Expression of the LETS protein was shown to be syntenic with GSR, which is coded by a gene on human chromosome 8 (41). No other linkage group was syntenic with LETS expression in primary and secondary human–mouse hybrid cells. The LETS protein was not syntenic with other external membrane proteins such as EMP-130 and EMP-195 (Figs. 1 and 2), whose genes have been assigned to human chromosomes 10 and 14, respectively (26,†). Evidence suggested that the EMP-175 protein in Figs. 1 and 2 is derived from EMP-195 (26,†). These data demonstrate that genes coding for external membrane proteins are located on different chromosomes rather than being clustered on the same chromosome in humans. In subclones of a hybrid positive for LETS and GSR, both markers continued to segregate together. This syntenic assignment was further supported by chromosome studies (Table 2), which demonstrated the concordant expression of the LETS protein, GSR, and chromosome 8. It is not currently possible from existing evidence to distinguish whether the LETS gene on chromosome 8 is the human structural gene or, less likely, a human control gene that is required for the expression of mouse, human, or both LETS proteins. Nevertheless, these results do demonstrate that a gene required for expression of the external membrane protein LETS is encoded on human chromosome 8.

Because the LETS protein has been used as a marker for tumorigenicity and cellular transformation, assignment of *LETS* to a human chromosome implicated in malignancy would be of considerable interest. Nonrandom karyotypic abnormalities of chromosome 8 have, in fact, been associated with various forms of cancer (42). Trisomy 8 has been correlated with various leukemias, including acute nonlymphocytic leukemia, chronic myelogenous leukemia in the blast phase, preleukemia, and other myeloproliferative disorders (42, 43). Translocations involving 8 and 21 are associated with acute myelogenous leukemia (44), and translocations of 8 and 14 have been found in Burkitt tumors (45). Other chromosomes, such as 1, 3, 5, 7, 9, 14, 17, 20, 21, and 22, are also nonrandomly associated with various forms of cancer with an overlap of some of the same neoplasias as observed for chromosome 8 abnormalities (42). These findings suggest that there is genetic information encoded on chromosome 8 that is implicated in cancer. The assignment of *LETS* to a human chromosome associated with malignancies may prove to be a significant observation.

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