Hu-*ets*-1 and Hu-*ets*-2 Genes Are Transposed in Acute Leukemias with (4;11) and (8;21) Translocations

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Human probes identifying the cellular homologs of the v-*ets* gene, Hu-*ets*-1 and Hu-*ets*-2, and two panels of rodent-human cell hybrids were used to study specific translocations occurring in acute leukemias. The human *ets*-1 gene was found to translocate from chromosome 11 to 4 in the t(4;11)(q21;23), a translocation characteristic of a subtype of leukemia that represents the expansion of a myeloid/lymphoid precursor cell. Similarly, the human *ets*-2 gene was found to translocate from chromosome 21 to chromosome 8 in the t(8;21)(q22;q22), a nonrandom translocation commonly found in patients with acute myeloid leukemia with morphology M2 (AML-M2). Both translocations are associated with expression different from the expression in normal lymphoid cells of *ets* genes, raising the possibility that these genes play a role in the pathogenesis of these leukemias.

A RESTRICTED NUMBER OF CHROMOSOME regions are implicated in human cancers, suggesting that they contain genes whose derangement is crucial to the establishment of neoplasia (*1*). Some translocations associated with hematopoietic tumors are known to be specific (*2*). Such cytogenetic accidents occurring to a hematopoietic cell at a particular differentiative stage and within a particular lineage (*3*) may initiate a cascade of molecular alterations that result in the leukemic transformation or may simply confer a proliferative advantage to that cell. Several proto-oncogenes (*4*) and other genes, such as those coding for immunoglobulins (*5*) and T-cell receptor (*6*), map at or near breakpoints of translocations and inversions characteristic in certain leukemias. Two examples of how chromosome translocations may involve some of these genes at the molecular level are (i) gene deregulation in Burkitt's lymphomas, with (8;14), (8;22), and (2;8) translocations involving the c-*myc* locus, and the heavy and light chain immunoglobulin (Ig) loci (*7*); and (ii) production of a chimeric protein in chronic myelogenous leukemia (CML) with the (9;22) translocation (Philadelphia chromosome). In the latter case a fusion product of *bcr-abl* genes is found (*8*).

We previously established that the *ets* sequence of the transforming avian erythroblastosis virus, E26 (*9*), has homologs in chicken, mouse, cat, and man. The *ets*-related sequences of human DNA, located on chromosome 11 (Hu-*ets*-1) and chromosome 21 (Hu-*ets*-2), are discontiguous except for a small overlap region encoding 14 amino acids, 12 of which are conserved between these two loci (*10*). Further, *ets*-1 and *ets*-2 behave as separate loci in the mouse, cat, and man (*11*).

The *ets* domains are situated within syntenic gene groups known to be conserved among the three diverse mammalian species (*11*). We have determined by in situ hybridization that human *ets*-2 is located in the 21q22.1-22.3 region (*11*), whereas human *ets*-1 was mapped to the 11q23-24 region (*12*). In addition, human *ets*-1 and *ets*-2 genes are transcriptionally active. The human *ets*-1 locus encodes a single messenger RNA (mRNA) of 6.8 kb, while the *ets*-2 locus encodes mRNA's of 4.7, 3.2, and 2.7 kb (*10*).

A number of leukemias of the myelomonocytic lineage show chromosome abnormalities in the very regions where the human-*ets* genes reside (*13*). The 11q23 region, where Hu-*ets*-1 is located, participates in a number of translocations involving a second variable chromosome, suggesting that this region must contain a critical gene (or genes) for neoplasia. These leukemias result from the expansion of elements of the myelomonocytic lineage with various degrees of maturation. We focused primarily on the t(4;11)(q21;q23) translocation (Fig. 1A) that represents a consistent feature of a subtype of leukemia that occurs particularly in infants and is thought to be congenital (*14*). The peculiar ultrastructural and immu-

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Table 1. Segregation of Hu-*ets*-1 gene in somatic cell hybrids derived from A3 fibroblasts × t(4;11) leukemic cells. Cell hybrids have been obtained by fusing Chinese hamster fibroblasts (A3) and leukemic cells containing the t(4;11)(q21;q23) translocation using the Sendai virus protocol (*31*). Adherent (hybrid) cells were selected in HAT medium; chromosome analysis was performed on R-banded metaphases spreads after heat denaturation. The isoenzyme markers, lactate dehydrogenase A (LDHA) in the 11p12.03-12.08 region and phosphoglucomutase-2 (PGM2) in the 4p14-q12 region, have been determined by Cellogel electrophoresis as described (*32*). A human DNA probe for the calcitonin growth factor–related peptide (CGFR) on the 11pter-q12 region completed the identification of the hybrids (*33*).

	Human chromosomes																												
Hybrid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	4q-	11q+	LDHA	PGM2	CGFR	Hu-ets-1
5B	+		+			+		+	+	+		+			+	+	+		+						+	+	+	+	nd	+
11B			+			+	+	+	+	±		+	+		+	+	+		+	+		+	+		+		-	+	nd	+
17A	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+		+	+	+	+		+		-	+	-	+
27B			+					+		+		+	+	+		+	+	+	+	+		●				+	+	-	+	-
30A											+						+				+	+					+	-	+	+
3B	+				+		+			+	+				+	+	+	±					+		+		+	+	+	+
12B	+		+	+	+	+	+	+		+		+			+	+	+	+	+		+	+				+				-

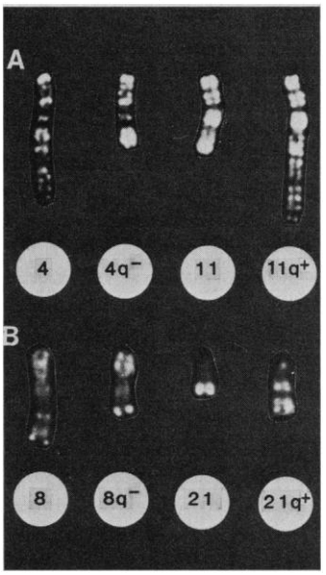

Fig. 1. Partial karyotype analysis showing the normal and recombinant chromosomes resulting from (A) the reciprocal translocation t(4;11)(q21;q23) and (B) the reciprocal translocation t(8;21)(q22;q22).

nologic characteristics of these leukemic blasts implicate a bone marrow precursor with the ability to differentiate either toward the myeloid or the lymphoid lineage. The 21q22 region where Hu-*ets*-2 resides is involved in the translocation (8;21)(q22;q22) (Fig. 1B) commonly found in acute myelogenous leukemia with morphology M2 (*15*). This region (21q22) is also implicated in Down's syndrome (*16*). Patients with three (or more) chromosomes 21 show a marked incidence of acute leukemia, particularly acute nonlymphocytic leukemia (ANLL) (*17*), whereas infants may present benign, reversible leukemoid reactions. This suggests that trisomy 21 is a predisposing factor for leukemic transformation.

In this report, we demonstrate that both Hu-*ets*-1 and Hu-*ets*-2 genes translocate from their normal position as a consequence of translocations involving the 11q23 and 21q22 breakpoints. This conclusion is based on analysis of two panels of somatic cell hybrids retaining either the normal or the recombinant chromosomes deriving from the (4;11) and (8;21) translocation (Fig. 1, A and B).

Chinese hamster fibroblasts (A3) were fused with an established leukemia cell line, RS4;11 (*18*), presenting the (4;11)(q21;q23) translocation. A number of cell hybrids (Table 1) have been isolated in selective HAT (hypoxanthine aminopterin thymidine) medium and subsequently identified cytogenetically by the R-banding technique and assayed biochemically for the presence of markers on chromosomes 4 and 11; the markers used were lactate dehydrogenase A (LDHA) for chromosome 11 and phosphoglucomutase-2 (PGM2) for chromosome 4. The calcitonin growth factor–related peptide gene located in the 11pter-11q12 region was detected by a specific DNA probe. The human *ets*-1 probe RD6K (*10*) was used to identify Hu-*ets*-1 sequences in DNA isolated from these cell hybrids.

The human 3.0-kb Pst I fragment, identified by the RD6K probe, is present in hybrids containing either the intact 11 or the recombinant $4q^-$ chromosomes (Fig. 2, lanes 3, 5, 6, and 7). The same is true for a 6.2-kb Eco RI fragment. This experiment left unresolved whether the chromosomal breakpoint occurs within or outside of the Hu-*ets*-1 gene. Nevertheless, Southern blot analysis with several restriction enzymes of DNA's from three t(4;11) leukemias from the RS4;11 cell line, two (9;11)(p22;q23) leukemias [acute myelomonocytic leukemia (AMMoL-M4) and acute monocytic leukemia (AMoL-M5), respectively], and of the myelomonocytic cell line HL-92 (*19*) containing the deletion (11)(q23) did not demonstrate any rearrangements of Hu-*ets*-1 (*20*).

We used a similar approach to investigate whether the Hu-*ets*-2 gene translocates in t(8;21) AML-M2 leukemia. Somatic cell hybrids containing recombinant derivatives $8q^-$ and $21q^+$ of the t(8;21) leukemia were isolated (Table 2). This panel of cell hybrids has been characterized by extensive segregation analysis; in addition, isozyme analysis was carried out for glutathione reductase activity (GSR) for chromosome 8, soluble superoxide dismutase (SOD-1) for chromosome 21, and probes for c-*myc* (8q24) and c-*mos* (8q22) genes (*21–24*).

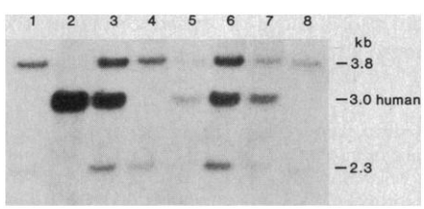

Fig. 2. Southern blot analysis of human lymphocytes, A3 Chinese hamster fibroblasts, and somatic cell hybrids derived from fusion A3 × t(4;11) leukemic cells. The DNA's were digested with Pst I and subjected to electrophoresis in an 0.8 percent agarose gel. Blot hybridization analysis with a human *ets*-1–specific (RD6K) probe (*10*) was carried out under stringent conditions [hybridization in 50 percent formamide, 5× SSC (1× SSC is 0.15*M* NaCl and 0.015*M* sodium citrate), 42°C; washings in 2× SSC, 0.5 percent sodium dodecyl sulfate at 65°C, 0.1× SSC at 25°C]. The human 3.0-kb Pst I fragment was detected in hybrids containing either the chromosome 11 or $4q^-$. (Lane 1) A3 fibroblasts; (lane 2) human lymphocytes; (lane 3) hybrid 30A (chromosome 11); (lane 4) hybrid 27B (chromosome $11q^+$); (lane 5) hybrid 5B(chromosome $4q^-$ and $11q^+$); (lane 6) hybrid 11B (chromosome $4q^-$); (lane 7) hybrid 17A (chromosome 4 and $4q^-$); (lane 8) hybrid 12B (chromosome 4 and $11q^+$).

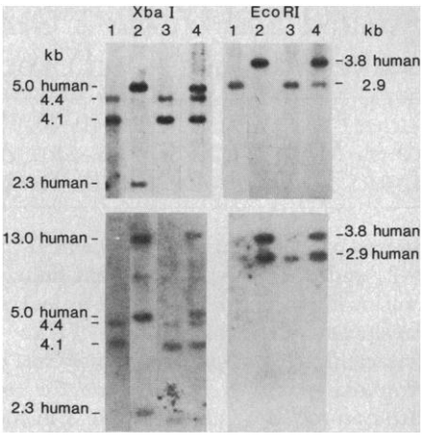

Fig. 3. Southern blot analysis of human lymphocytes, CHO Chinese hamster cells, and somatic cell hybrids derived from fusion CHO × t(8;21) leukemic cells. The DNA's were digested with Xba I and Eco RI and subjected to electrophoresis in an 0.8 percent agarose gel. Blot hybridization analyses with the human *ets*-2–specific probes H33 (top) and cDNA 14 (bottom) were carried out as described (legend to Fig. 2). (Lane 1) Chinese hamster cells; (lane 2) human lymphocytes; (lane 3) 21-8Ab5-23 ($21q^+$) hybrid; (lane 4) 13b1S816 ($8q^-$) hybrid. The Eco RI 3.8-kb and the Xba I 5.0-kb human fragments were detectable only in the $8q^-$ hybrid. The cDNA 14 probe distinguishes an Eco R1 2.9-kb human fragment overlapping with the hamster fragment and an Xba I 13-kb fragment.

DNA extracted from these cell hybrids was subjected to Southern blot analysis with the use of a human *ets*-2 genomic probe (H33) and an *ets*-2 probe designated complementary DNA (cDNA) 14 (*10*). The 3.8-kb Eco RI fragment of the Hu-*ets*-2 gene is present in the cell hybrid containing the recombinant chromosome $8q^-$ but not in the hybrid containing the chromosome $21q^+$, indicating that the *ets*-2 gene had been transposed from chromosome 21 to chromosome 8 (Fig. 3). Similarly, the Bam HI 6.8-kb fragment, the Pst I 12-kb fragment, and the Hind III 3.5-kb fragment are present in the hybrid with chromosome $8q^-$ but not in the one with chromosome $21q^+$ (data not shown). In the Xba I digest (Fig. 3, bottom) only the human 5.0-kb fragment but not the 2.3-kb fragment is detectable in the $8q^-$–containing hybrid. Thus, the Xba I site of the Hu-*ets*-2 gene can be eliminated as a consequence of translocation. Similar results were obtained using the same blots with the cDNA 14 probe. With this probe, we detected a 2.9-kb Eco RI fragment and an additional 13-kb Xba I fragment associated only with the cell hybrid containing the $8q^-$ chromosome. We

can therefore conclude that at least 20.3 kb of the Hu-*ets*-2 gene was translocated to chromosome 8.

DNA's from eight t(8;21) AML-M2 patients were subjected to Southern blot analysis with the same restriction enzymes as above and no Hu-*ets*-2 gene rearrangements were detected. Moreover, the ^{3}H-labeled Hu-*ets*-2 probe, H33, hybridized specifically to the 8q$^-$ chromosome of fresh leukemic cells from t(8;21) AML-M2 patients (*29*). In situ hybridization analysis with the v-*ets* 1.2-kb Bgl II fragment (*9*) of fresh leukemic cells having another translocation (9;11) (p21;q23), which involves the 11q23 region, shows the translocation of *ets* sequences from chromosome 11 to chromosome 9 (*30*). This translocation is usually associated with AMMoL-M4 and AMoL-M5 (*13*).

We next sought to determine whether the translocations of human *ets*-1 and *ets*-2 affected the transcription patterns of these genes. This could only be performed on a limited number of patients who were characterized cytogenetically. In addition, fresh bone marrow blast specimens are needed to yield intact RNA. We have, nonetheless, been able to assay for the expression of mRNA in fresh leukemic samples. Total RNA was isolated from leukemic cells and compared to normal human lymphocyte RNA in Northern blot experiments. In one AUL t(4;11) leukemia we observed only low levels of Hu-*ets*-1 mRNA (Fig. 4A); similar results were also obtained using RNA from the RS4;11 cell line. In two t(8;21) AML-M2 leukemias we observed the absence of some human *ets*-2 mRNA species present in normal control lymphocytes (Fig. 4B) and known to be present in several other tissues and cell lines of human origin (*10*); these were the 4.7-kb and 3.2-kb mRNA species in one patient sample, and the 4.7-kb species in the other. In addition, the expression of Hu-*ets*-2 gene in the t(8;21) cells is much lower than in control lymphocytes. It is possible that the altered expression of *ets*-1 and *ets*-2 genes is a consequence of the translocations observed. Alternatively, this pattern could be typical of the myeloid precursor cells, the normal cell counterparts of these leukemic blasts. Myeloid precursor cells, rather than lymphocytes, would be the ideal controls for these studies, but they are virtually impossible to identify and isolate from normal bone marrow.

These studies indicate that the Hu-*ets*-2 gene translocates to the 8q$^-$chromosome, suggested (*2*) to be the critical recombinant chromosome in the t(8;21) translocation on the circumstantial cytogenetic evidence that this chromosome is the one constant in both the simple and complex translocations. Another proto-oncogene, c-*mos*, located at the 8q22 breakpoint site, does not translocate to the other recombinant chromosome, 21q$^+$ (*23*).

The 11q23 region is also believed to be critical for myeloid transformation, since this region is a constant one, participating in translocations involving a second variable chromosome. It has been suggested (*2*) that translocations can bring together two types of genes, one related to growth control (proto-oncogene) in certain hematopoietic cells in a specific differentiative stage, and another gene, coding for a protein critical to that cell at that stage. In this context, 11q23 can be postulated to contain the locus for a growth-regulatory factor (that is, Hu-*ets*-1 product), and the regions of the other chromosome (4q21 or 9p22) the loci for myeloid stage-specific genes. The molecular characterization of the breakpoints, as well as the identification of the human *ets*-1 and *ets*-2 gene products, are necessary to establish whether these genes play a crucial role in the development of acute leukemias with the cytogenetic abnormalities discussed.

Table 2. Segregation of Hu-*ets*-2 gene in somatic cell hybrids derived from CHO × t(8;21) leukemic cells. The 706B clone 17 (*19*) and 72532X-6 (*24*) are Chinese hamster ovary (CHO)–human hybrids containing chromosomes 8 and 21, respectively (*21–22*). The other hybrids were derived from the fusion of CHO mutants and t(8;21) AML-M2 cells separated from erythrocytes and mature granulocytes on Ficoll-Hypaque gradients. The 21-8Ab5-23 hybrid contains the 21q$^+$ derivative and was obtained from a CHO Gly$^-$ B parent defective in glycine metabolism (*23*), whereas the 13b1S816 hybrid contains the 8q$^-$ derivative and was obtained from a CHO Ade$^-$ C parent defective in purine metabolism (*24*). Cell hybrids were selected in F12D medium (*25*) and dialyzed serum (6 percent). Chromosome segregation was achieved using a bromodeoxyuridine selection method (*26*). Chromosomes were identified with Giemsa/trypsin banding and Giemsa 11 staining (*27*). Isozyme analysis for glutathione reductase (GSR, in the 8p21.1 region) and soluble superoxide dismutase (SOD1, in the 21q22.1 region), were performed as described (*32*). The human c-*mos* (8q22) and c-*myc* cDNA (8q24) probes (*28*) allowed a more precise characterization of the hybrids.

Hybrid	Human chromosomes				GSR	SODI	c-*myc*	c-*mos*	Hu-*ets*-2
	8	21	8q$^-$	21q$^+$					
706BC117	+				+	−	+	+	−
72532X-6		+			−	+	−	−	+
13b1S816			+		+	−	−	+	+
21-8Ab5-23				+	−	+	+	−	−

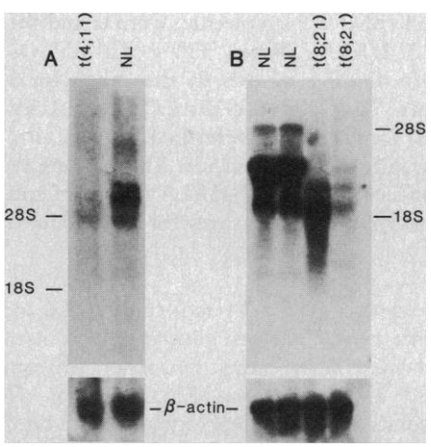

Fig. 4. Northern blot analysis of total RNA extracted from (A) blast cells derived from a t(4;11) AUL patient and normal lymphocytes (NL), and (B) blast cells derived from two t(8;21) AML-M2 patients and normal lymphocytes. In all cases RNA was extracted by the guanidinium–cesium chloride method and resolved on 1.2 percent formaldehyde-agarose gels (*34*). Hybridization was performed under stringent conditions (as described in the legend to Fig. 2) with the use of (A) the *ets*-1 (RD6K) probe and (B) the *ets*-2 (H33) probe. The same blots (bottom) have been rehybridized with a β-actin cDNA probe to compare the amount of RNA in these samples.

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Binding of HTLV-III/LAV to T4$^+$ T Cells by a Complex of the 110K Viral Protein and the T4 Molecule

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Human T-lymphotropic virus type III (HTLV-III) or lymphadenopathy-associated virus (LAV) is tropic for human T cells with the helper-inducer phenotype, as defined by reactivity with monoclonal antibodies specific for the T4 molecule. Treatment of T4$^+$ T cells with monoclonal antibodies to T4 antigen blocks HTLV-III/LAV binding, syncytia formation, and infectivity. Thus, it has been inferred that the T4 molecule itself is a virus receptor. In the present studies, the surfaces of T4$^+$ T cells were labeled radioactively, and then the cells were exposed to virus. After the cells were lysed, HTLV-III/LAV antibodies were found to precipitate a surface protein with a molecular weight of 58,000 (58K). By blocking and absorption experiments, this 58K protein was identified as the T4 molecule. No cell-surface structures other than the T4 molecule were involved in the antibody-antigen complex formation. Two monoclonal antibodies, each reactive with a separate epitope of the T4 molecule, were tested for their binding capacities in the presence of HTLV-III/LAV. When HTLV-III/LAV was bound to T4$^+$ T cells, the virus blocked the binding of one of the monoclonal antibodies, T4A (OKT4A), but not of the other, T4 (OKT4). When HTLV-III/LAV was internally radiolabeled and bound to T4$^+$ T cells which were then lysed, a viral glycoprotein of 110K (gp110) coprecipitated with the T4 molecule. The binding of gp110 to the T4 molecule may thus be a major factor in HTLV-III/LAV tropism and may prove useful in developing therapeutic or preventive measures for the acquired immune deficiency syndrome.

Infection with the retrovirus referred to as human T-lymphotropic virus type III (HTLV-III) or lymphadenopathy-associated virus (LAV) results in a numerical and functional depletion of T helper/inducer cells (*1–3*). In its most severe form, infection is clinically manifest as susceptibility to opportunistic infections or malignancies and is known as the acquired immune deficiency syndrome (AIDS) (*1, 3–5*). The essential immunologic features of AIDS can be reproduced in cultures of normal human lymphocytes where HTLV-III/LAV infects, replicates in, and ultimately depletes T cells with the helper/inducer phenotype as defined by reactivity with monoclonal antibodies (T4, Leu 3, and CD4) (*1, 3–8*).

Apparently the T4 molecule on the cell surface of T helper/inducer cells is itself involved in the tropic interaction between T4$^+$ T cells and virus. Klatzman *et al.* (*7*) reported that viral replication was inhibited in vitro by incubating T cells with T4 monoclonal antibody (mAb), which indicates involvement of the T4 antigen in some part of the replication cycle. Dalgleish *et al.* (*8*) constructed a virus pseudotype between HTLV-III/LAV and vesicular stomatitis virus and demonstrated that treating target cells with CD4 mAb inhibited virus replication and syncytia formation (*8*). If the role of HTLV-III/LAV virus in the pseudotype virus is to permit virus penetration (*9*), these experiments provide more direct evidence that the T4 molecule is a virus receptor. In direct-binding studies, we have demonstrated that cell-surface binding of HTLV-III/LAV and T4A mAb reciprocally inhibit each other and that treating lymphocytes with T4A mAb before virus inoculation inhibits infection and virus replication (*10*). In the present studies, we demonstrate at a molecular level the coprecipitation of virus and the T4 molecule and show which virus structure or structures participate in the binding reaction.

In selecting a strategy for these experiments, we considered some results of binding studies (Table 1). If phytohemagglutinin (PHA)-stimulated normal human lymphocytes are treated with T4A mAb before being exposed to HTLV-III, binding of virus to these cells is inhibited. Treatment with T4 mAb, which binds to a separate site (epitope) of the T4 molecule (*11*), does not inhibit HTLV-III/LAV binding. Conversely, if the cells are first treated with HTLV-III/LAV, the binding of T4A mAb is inhibited but the binding of T4 mAb is not. Binding and binding inhibition results are the same whether performed on the T4$^+$ T-cell line CEM, on PHA-stimulated lymphocytes, or on unstimulated lymphocytes (*10*). Three prototype strains of virus (HTLV-III, LAV, and CDC-451) grown in PHA-stimulated lymphocytes or the H9 or CEM cell lines were tested, and all displayed T4 binding. [Reciprocal inhibition did not occur with any of a panel of other mAb's, including T3, T11, T8, Leu 8, T17, T10, Ia, Tac, 4F2, and T9, and inhibition with T4A mAb was not due to a reaction with the virus rather than with cells (*10*)]. Thus, with respect to a putative HTLV-III/LAV protein–T4 complex, the epitope recognized by T4 mAb on the complex should be accessible whereas the T4A epitope may not be.

A continuous human T4$^+$ T-cell line

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