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Genomic structure of HTLV (human T-cell leukemia virus): detection of defective genome and its amplification in MT-2 cells

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We studied the genomic structure of human T-cell leukemia virus (HTLV) in the HTLV producer cell line MT-2. Southern blotting revealed that at least eight HTLV proviruses were integrated in the chromosomes of MT-2 cells. The genomic structure of these proviruses was analyzed using fragments of cloned HTLV that were specific to *gag, pol, env, pXs* and U_3R genes as probes. We have identified a complete genome of HTLV in MT-2 (non-defective type). However, seven of the eight proviruses had defective genomes. Provirus T2-a contains only the U_3R (LTR) of HTLV and T2-b corresponds to the non-defective genome. T2-c possesses only a portion of *env,* and *pXs* and U_3R. T2-d consists of *gag, pol,* part of *env* and U_3R. On the other hand, T2-e, f, g and h consist of *gag, pXs* and U_3R. Northern blotting experiments with mRNA from MT-2 cells supported the evidence of amplification of the *gag-pXs* gene of HTLV. 26S mRNA is considered to be a subgenomic species of 35S RNA. 32S mRNA may represent the T2-d provirus which lacks a portion of *env* and *pXs,* while 20S mRNA was a subgenomic species. The *gag-pXs* gene may correspond to 24S mRNA, the amount which was amplified in MT-2 cells.

Key words: human leukemia virus/Southern blotting/Northern blotting/defective genome/gene amplification

Introduction

C-type RNA tumor viruses are involved in naturally occurring neoplasms such as sarcomas, leukemia and lymphomas of various species of vertebrates (Aaronson and Stephenson, 1976). In 1980, Poiesz *et al.* first isolated a human type C retrovirus from sporadic adult T-cell leukemia-lymphoma patients in the USA (Poiesz *et al.*, 1980, 1981). This retrovirus, designated human T-cell leukemia virus (HTLV), was also isolated from a human T-cell line established from a patient with adult T-cell leukemia (ATL) in Japan by Hinuma *et al.* (Hinuma *et al.*, 1981; Miyoshi *et al.*, 1981; Yoshida *et al.*, 1982) and subsequently from many patients from other regions of the world (Popovic *et al.*, 1983).

ATL is a clinical entity of human T-cell malignancy, which was first described by Takatsuki *et al.* in 1977. It is endemic in the southwestern part of Japan (Takatsuki *et al.*, 1977; Uchiyama *et al.*, 1977) and also in the Caribbean area (Catovsky *et al.*, 1982). Seroepidemiologic studies revealed that almost all of the ATL patients possess antibodies reactive with cell lines established from ATL patients (Hinuma *et al.*, 1981, 1982; Kalyanaraman *et al.*, 1981a; Posner *et al.*, 1981). These antigens, termed ATL-associated antigens (ATLA), were found to include HTLV core proteins p19, p24 and glycosylated envelope proteins gp68, and gp46 (Kalyanaraman *et al.*, 1981b; Robert-Guroff *et al.*, 1981; Yamamoto and Hinuma, 1982; Yamamoto *et al.*, 1982; Yoshida *et al.*, 1982; Manzari *et al.*, 1983; Hattori *et al.*, 1983; Kobayashi *et al.*, 1984).

The existence of HTLV proviruses was demonstrated in peripheral blood leukocytes (PBL) of ATL patients without exception, but not in PBL of healthy adults (Reitz *et al.*, 1981; Yoshida *et al.*, 1982; Manzari *et al.*, 1983). All the patients so far tested possessed at least one copy of the HTLV provirus in their PBL, while the sites of integration apparently differ among individual patients. Thus, genomic information carried in HTLV is considered to be exogenous to humans.

Recently, Seiki *et al.* (1983) cloned a provirus of HTLV from PBL of an ATL patient, and reported that the genome apparently lacks known oncogenes. HTLV consists of *gag, pol, env* and *pXs* flanked by LTRs. The structure of HTLV apparently resembles those of animal leukemia viruses such as ALV or MuLV. However, there were almost no sequence homologies between HTLV and known leukemia viruses, except that a portion of p24 resembles that of BoLV (Poiesz *et al.*, 1980; Oroszlan *et al.*, 1982). Thus, the mechanism of cellular transformation by HTLV may mimic those of known leukemia viruses, even though the function of *pXs* is not known yet. Data so far accumulated strongly suggest that HTLV is a causative agent of ATL. To understand the molecular mechanism of cellular transformation by HTLV, we have analysed the structure of HTLV in the HTLV producer cell line MT-2. We found that the MT-2 cell possesses at least eight individual HTLV proviruses but seven of them are defective.

Results

Restriction endonuclease map of cloned HTLV

We first cloned a non-defective HTLV from the PBL of an ATL patient. Figure 1 shows the restriction endonuclease map of the cloned HTLV, which is nearly identical to those described by Seiki *et al.* (1983). We prepared several fragments of HTLV that correspond to *gag, pol, env, pXs* and U_3R regions following the designation of Seiki *et al.*. Thus the *Sma*I-*Sma*I fragment (1.1 kb), *Hind*III-*Hind*III fragment (2.0 kb), *Bam*HI-*Xho*I fragment (0.67 kb), *Bam*HI-*Xho*I fragment (0.4 kb), *Pst*I-*Cla*I fragment (0.75 kb) and *Sma*I-*Taq*I fragment (0.45 kb) shown in Figure 1 were used as specific probes respectively for *gag, pol* the 5′ portion of *env*, the 3′ portion of *env, pXs* and U_3R.

Southern blotting of MT-2 DNA with HTLV fragments

DNA of MT-2 cells, prepared as described in Materials and methods, was completely digested with restriction endonuclease *Eco*RI and separated on 0.8% agarose gel. After gel electrophoresis, DNA in the gel was transferred to nylon

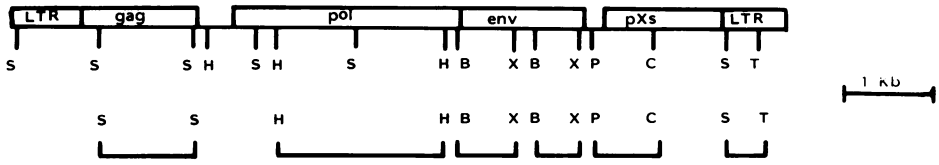

Fig. 1. Restriction endonuclease map of non-defective HTLV. S: *Sma*I; H: *Hind*III; B: *Bam*HI; X: *Xho*I; P: *Pst*I; C: *Cla*I; T: *Taq*I.

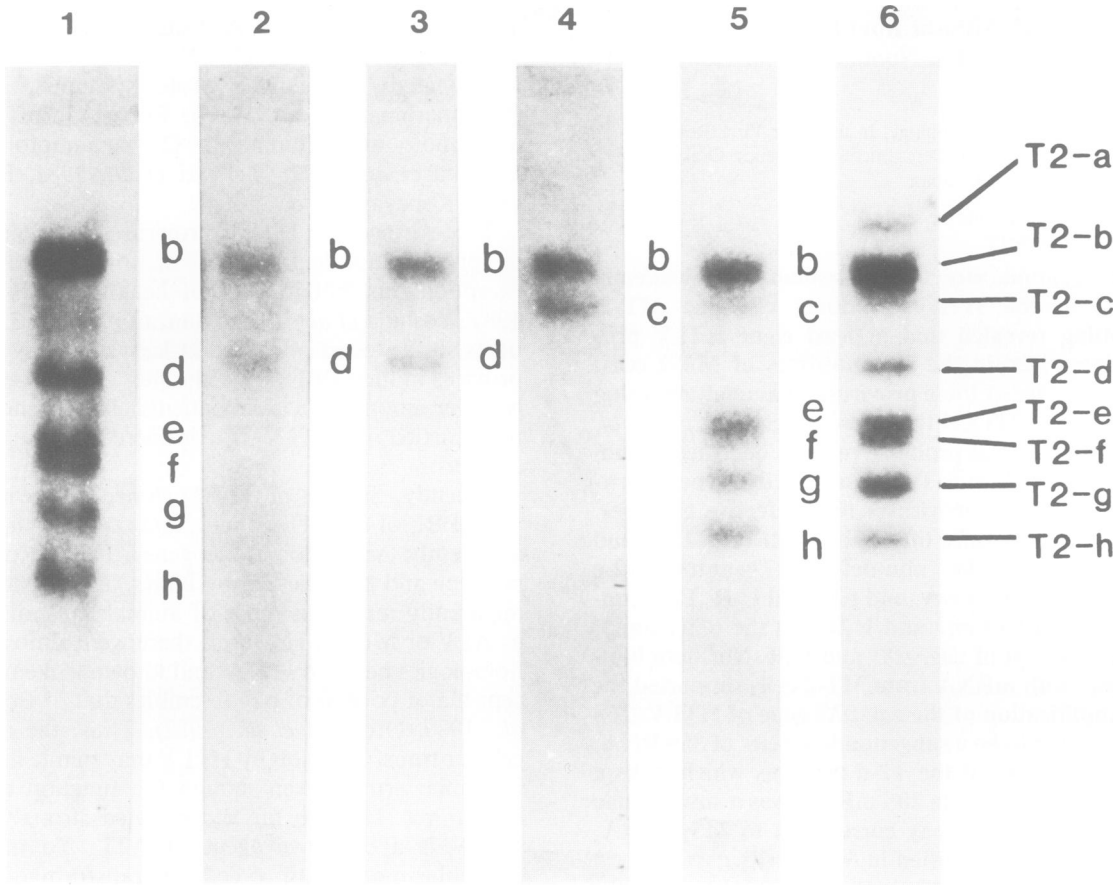

Fig. 2. Southern blotting of MT-2 DNA with HTLV-specific fragments. Specific fragments of HTLV shown in Figure 1 were hybridized with *Eco*RI-digested MT-2 DNA as described in Materials and methods. Fragments used were **(1)** *gag*, **(2)** *pol*, **(3)** 5′ end of *env*, **(4)** 3′ end of *env*, **(5)** *pXs*, **(6)** U_3R.

membrane filters and Southern blotting was performed with ^{32}P-labeled HTLV fragments. When the U_3R portion was used as a probe (Figure 2, lane 6) at least eight individual bands were detected. We have tentatively termed these HTLV proviruses as T2-a, T2-b, ... T2-g, and T2-h according to their size as shown in Figure 2. It is clear from Figure 2 that T2-a hybridizes only to U_3R but not to *gag, pol, env* or *pXs*. T2-b hybridizes with all the fragments prepared as shown in Figure 1. T2-c hybridizes to U_3R, *pXs* and the 3′ end of *env*. T2-d hybridizes to U_3R, the 5′ portion of *env, pol* and *gag* but shows no homology with *pXs* or the 3′ portion of *env*, while T2-e, T2-f, T2-g and T2-h show homology only to *gag, pXs* and U_3R. The results of Figure 2 are summarized in Table I.

Table I. Structure of HTLV proviruses in MT-2 cells

Proviruses	Size of *Eco*RI digested (kb)	*gag*	*pol*	*env* 5′	*env* 3′	*pXs*	U_3R
T2-a	30	−	−	−	−	−	+
T2-b	18.0	+	+	+	+	+	+
T2-c	14.0	−	−	−	+	+	+
T2-d	9.6	+	+	+	−	−	+
T2-e	7.5	+	−	−	−	+	+
T2-f	6.9	+	−	−	−	+	+
T2-g	5.7	+	−	−	−	+	+
T2-h	4.8	+	−	−	−	+	+

Northern blotting of cytoplasmic mRNA of MT-2 with HTLV fragments

We next made Northern blots of cytoplasmic poly(A)-containing mRNA of MT-2 cells prepared using guanidinium thiocyanate as described in Materials and methods. The cytoplasmic mRNA thus obtained was enriched for poly(A) content by two cycle passages through oligo(dT)-cellulose columns. 0.1 μg of mRNA was separated by 1% agarose gel electrophoresis after denaturing with glyoxal. RNAs in the gel were then transferred to nylon-membrane filters and Northern blotting was performed as described by Thomas (1980). With the U_3R portion as a probe, 35S, 32S, 26S, 24S and 20S mRNAs hybridized (Figure 3, lane 6). The 24S RNA was the predominant species. Using different HTLV-specific probes it

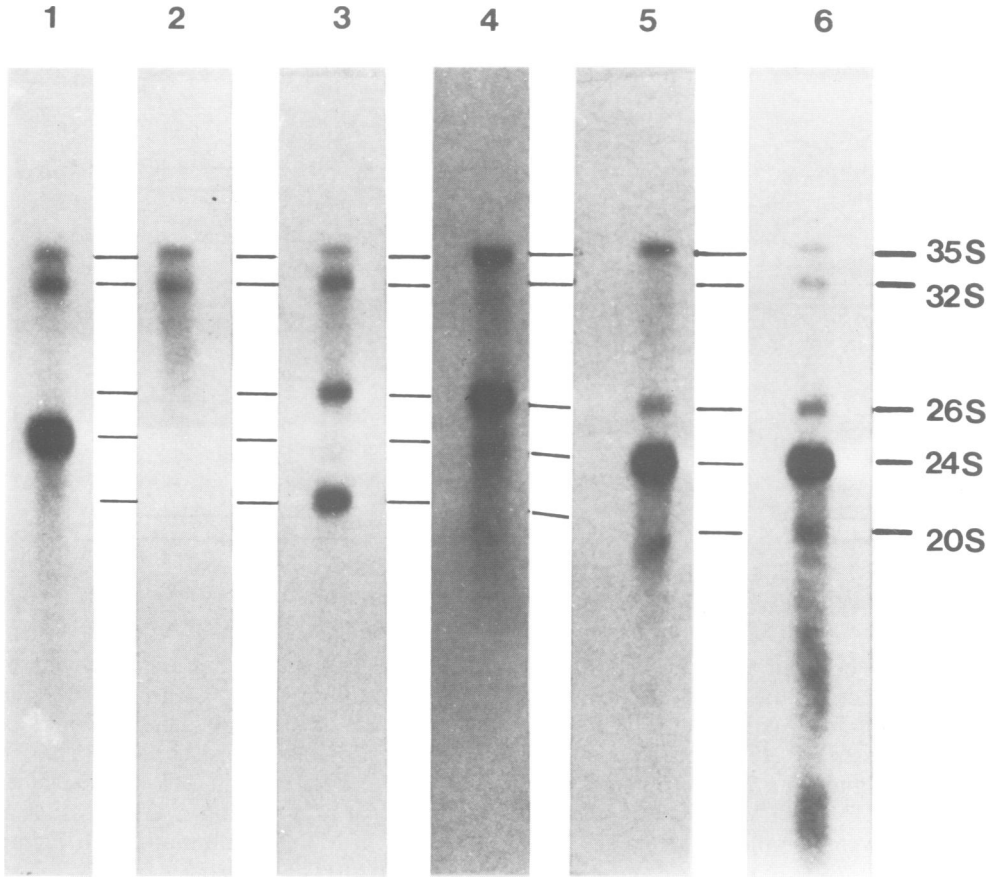

Fig. 3. Northern blotting of cytoplasmic poly(A) mRNA of MT-2. Specific fragments of HTLV shown in Figure 1 were hybridized with poly(A)-containing cytoplasmic mRNA of MT-2. Fragments used were **(1)** *gag*; **(2)** *pol*; **(3)** 5′ portion of *env*: **(4)** 3′ portion of *env*; **(5)** *pXs*; **(6)** U_3R.

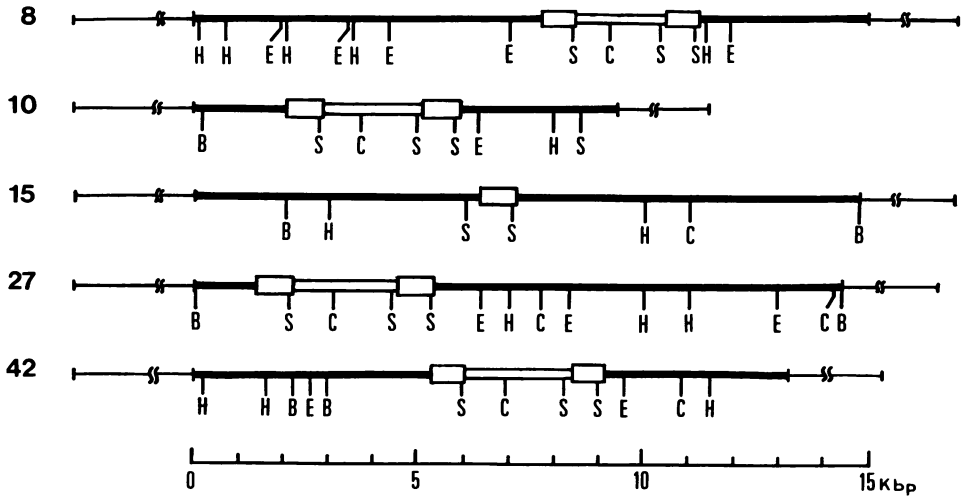

Fig. 4. Restriction endonuclease map of HTLV provirus clone from MT-2. Open bar represents the portions which hybridize to the fragment of HTLV, closed bar represents the cellular flanking sequences. E: *Eco*RI; S: *Sma*I; C: *Cla*I; H: *Hind*III; B: *Bam*HI.

is clear that 35S RNA hybridizes to *gag, pol, env, pXs* and U_3R, thus representing the helper type, non-defective genome of HTLV. 26S mRNA hybridizes to *env, pXs* and U_3R. 32S mRNA hybridized to *gag, pol,* the 5′ portion of *env* and U_3R. 20S mRNA hybridized to the 5′ portion of *env* and U_3R only. 24S mRNA on the other hand hybridizes to *gag, pXs* and U_3R.

Structure of defective type HTLV

To characterize their structure more precisely we cloned the HTLV proviruses from MT-2. The DNA from MT-2 was partially digested with restriction endonuclease *Sau*3A to give an average size of DNA between 10 and 20 kb, and ligated with the vector Charon 28. Figure 4 shows the HTLV clone. We have identified so far five individual HTLV clones that hybridize to the U_3R portion of HTLV. Clone #15 consists of 14.7 kb and hybridizes to only the U_3R probe of HTLV which lacks the *Eco*RI site. Clones #8, 10, 27 and 42 hybridize to the U_3R probe at two different places, and the

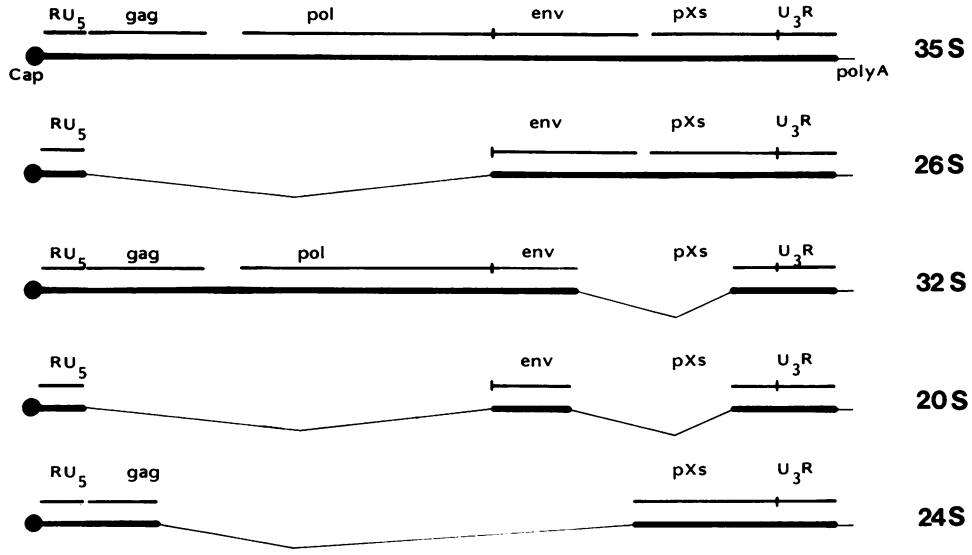

Fig. 5. Possible genomic structure of HTLV in MT-2.

gag and *pX* portion residing between these two LTR portions. Cellular flanking sequences of these clones differ from each other; Clone #8 contains five *Eco*RI sites in which the 4.8-kb *Eco*RI fragment possesses the HTLV provirus. Clone #42 contains two *Eco*RI sites making a 7.0-kb fragment with a portion of HTLV provirus. The HTLV proviruses detected in clones #8, 10, 27 and 42 were completely identical.

Discussion

A human type C retrovirus HTLV was isolated from a cell line termed MT-2 (Miyoshi *et al.*, 1981), which was established from peripheral leukocytes of an ATL patient by co-cultivation with cord leukocytes. HTLV is exogenous to humans (Yoshida *et al.*, 1982). The association of HTLV genome with peripheral leukocytes of ATL patients strongly suggests that HTLV is closely related to this malignancy.

HTLV resembles known leukemic-type animal retroviruses that lack an apparent *'onc'* gene but possess the *gag, pol* and *env* genes indispensable for viral replication (Seiki *et al.*, 1983). The *gag* gene of HTLV codes for a polypeptide of 48 K (Pr 48^{gag}), which could be cleaved into three polypeptides of 14 K, 24 K and 10 K. From the sequence data it is clear that the predicted amino acid sequences of the amino terminus of 24 K is identical with the sequence of the p24 of ATLA (Oroszlan *et al.*, 1982). The *env* gene of HTLV was predicted to code for a 54-K polypeptide composed of 488 amino acids. Antibody prepared against the predicted *env* gene product of HTLV recognizes gp61 of ATLA in MT-2 cells (Hattori *et al.*, 1983).

We previously showed that gene products of HTLV mRNA translated *in vitro* cross-react with antibodies against ATLA (Kobayashi *et al.*, 1984). To understand the molecular mechanism of cellular transformation by this newly isolated HTLV we studied the genomic structure of HTLV in a HTLV producer cell line MT-2.

When DNA from MT-2 was completely digested with *Eco*RI, we could detect at least eight individual bands which hybridize to the U_3R probe of HTLV (Figure 2). As HTLV-I possesses no *Eco*RI site in its genome (Yoshida *et al.*, 1982; Seiki *et al.*, 1983; Wong-Staal *et al.*, 1983), these eight individual bands may represent individual HTLV proviruses. Our Southern and Northern blotting experiments clearly show that 24S HTLV genome could be transcribed from HTLV provirus T2-e, f, g and h. Thus, the amplification of 24S HTLV mRNA in MT-2 is explained by the amplified number of provirus copies. The possible genomic structure of HTLV in MT-2 is summarized in Figure 5. Previously, we found that HTLV particles from MT-2 contain 35S, 32S and 24S mRNA, in contrast, 26S and 20S mRNA were detected only in the cytoplasm of MT-2 (Kobayashi *et al.*, 1984). These findings, together with the data presented here, confirm the concept illustrated in Figure 5.

We have shown previously that 24S RNA of HTLV directs the synthesis of p28 of ATLA in a nuclease-treated rabbit reticulocyte system (Kobayashi *et al.*, 1984). p28, which was chiefly found on the membrane fraction of cells (Schneider *et al.*, 1984), is one of the main components of ATLA in MT-2 cells. The function and detailed structure of p28 is not known yet but p28 is immunoprecipitated with a monoclonal antibody GIN-7 that reacts with the *gag* gene products p19 and p53 of HTLV (Tanaka *et al.*, 1983). p19 resides in the amino terminus of *gag* genes (Manzari *et al.*, 1983; Seiki *et al.*, 1983) and p53, which was detected in the cellular matrix only (Yamamoto and Hinuma, 1982), is considered as a precursor polypeptide of the *gag* gene. Another polypeptide of the *gag* gene of HTLV is p24, which resides in the middle of the *gag* gene (Manzari *et al.*, 1983; Seiki *et al.*, 1983). Antibodies raised against p24 react with p53 but not with p28 (Kobayashi *et al.*, 1984). Thus, we postulate that 24S HTLV possesses only a portion of the *gag* gene, possibly a portion of p19 but not a portion of p24. Our preliminary results on the sequence of clone #42 support the idea that clone #42 contains complete sequences corresponding to p19 (data not shown). Previously it was speculated that p28 may be a possible precursor of p19, from the reactivity with monoclonal antibody GIN-7 (Tanaka *et al.*, 1983). However, when MT-2 cells were pulse-labeled (10 min) with [^{3}H]leucine, p53 could be chased for a relatively short time (< 3 h) while the labeling of p28 changed little during this chase period (data not shown), suggesting that p28 is not a precursor of p19.

This is the first report of a defective type of HTLV.

Although the biological functions as well as the significance of the amplification of 24S RNA of HTLV in MT-2 cells remain unclear, it is well known that most of the viral genomic information for cellular transformation *(onc)* resides in replication-defective retroviruses. Thus, p28 of ATLA encoded by 24S RNA of HTLV may be associated with the activity responsible for the cellular transformation. Further studies are underway to elucidate this point.

Materials and methods

Cells

The HTLV producer cell line MT-2 was maintained in RPMI 1640 supplemented with 10% fetal calf serum. The medium was changed twice a week.

Cloning of HTLV and preparation of specific fragments

A complete non-defective genome of HTLV was cloned into a vector Charon 28 at the *Bam*HI restriction endonuclease site. DNA for cloning was prepared from the PBL of an ATL patient. *Bam*HI completely digested DNA was used for cloning. A complementary DNA clone of HTLV (Shigesada *et al.*, unpublished) was used for screening of the complete HTLV genome. Cloning of HTLV proviruses from MT-2 was conducted as above except that *Sau*3A partially digested MT-2 DNA was used.

Preparation of chromosomal DNA

MT-2 cells were removed from medium and washed twice with PBS and lysed in STE (0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA), 0.5% SDS, 500 μg/ml pronase K (~1 ml/1 x 10^6 cells). After incubating at 37°C for 1 h, lysate was mixed with an equal volume of STE-saturated phenol and gently shaken for 5 min. Aqueous phase was recovered by centrifuging the mixture for 10 min at 2000 r.p.m. Aqueous phase was re-extracted with STE-saturated phenol and ethanol precipitated. Precipitates were recovered by centrifugation at 10 000 r.p.m. with a Beckman JA-20 rotor at 4°C for 30 min. They were washed once with cold 70% EtOH, air dried, and then dissolved in H_2O. DNA was then treated with RNase (100 μg/ml) at 37°C for 45 min followed by pronase K digestion (500 μg/ml) at 37°C for 45 min. DNA was extracted again with STE-saturated phenol and precipitated with EtOH. DNA thus obtained was dissolved in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and stored at 4°C. Molecular size of the DNA thus obtained was >50 kb.

Preparation of mRNA

Cytoplasmic poly(A)-containing mRNA was obtained according to the methods described by Chirgwin (Chirgwin *et al.*, 1979) using guanidinium thiocyanate. Poly(A)-containing mRNA was selected by passing through two cycles of oligo(dT)-cellulose (P.L. Biochem type 7) column (Aviv and Leder, 1972). mRNA thus obtained was dissolved in 50% EtOH and stored at −20°C.

Southern blotting and Northern blotting

*Eco*RI completely digested DNA (10 μg) was separated on 0.8% agarose gel in TAE (40 mM Tris-acetate, pH 8.0, 2 mM EDTA). Electrophoresis was conducted at 20 V constant for 16 h. After electrophoresis DNA on the gel was stained with EtBr and transferred to nylon membrane filter (Pall BIODYNE). *Hind*III-digested λ DNA was used as a molecular size marker.

mRNA (0.1 μg) was separated on 1% agarose gel electrophoresis under conditions using glyoxal as described by McMaster and Carmichael (1977). Electrophoresis was conducted at 70 V constant. After electrophoresis, RNA in the gel was transferred to a nylon membrane filter. 28S and 18S rRNAs prepared from MT-2 cells were used as molecular size markers.

DNA-DNA hybridization was performed in 5 x Denhardt, 0.9 M NaCl, 50 mM phosphate buffer pH 7.7, 5 mM EDTA, 0.2% SDS, at 68°C. RNA-DNA hybridization was performed as described by Thomas (1980).

Preparation of nick-translated ^{32}P-labeled probes

^{32}P-Labeled nick-translated probes were prepared using a nick-translation kit purchased from Amersham (Amersham, Japan). [^{32}P]dCTP (Amersham >400 Ci/mmol) was used for labeling DNAs. 5 − 10 x 10^6 c.p.m./ml of labeled DNA (4 x 10^8 d.p.m./μg DNA) was used for hybridization.

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