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Translation of HTLV (human T-cell leukemia virus) RNA in a nuclease-treated rabbit reticulocyte system

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A human type-C retrovirus, designated HTLV (human T-cell leukemia virus), was isolated from the HTLV producer cell line MT-2. Agarose gel electrophoresis analysis ^{32}P-labeled HTLV$_{MT-2}$ virion RNA revealed that HTLV$_{MT-2}$ virion RNA consists mainly of 24S and small amounts of 35S and 32S RNAs. The 24S HTLV$_{MT-2}$ virion RNA and unfractionated HTLV$_{MT-2}$ virion RNA were translated in a rabbit reticulocyte lysate system *in vitro*. The predominant polypeptide synthesized from 24S RNA had an apparent mol. wt. of 28 000 (28 K); unfractionated HTLV$_{MT-2}$ virion RNA directed the synthesis of 53 000 (53 K), 33 000 (33 K) and 28 000 (28 K) polypeptides as main components. Most of the polypeptides synthesised *in vitro* by translation of HTLV$_{MT-2}$ virion RNAs possess the same sizes as the proteins formerly designated as ATLA (ATL-associated antigen) in SDS-polyacrylamide gel electrophoresis and immunologically precipitated with sera of ATL patients. Therefore, the antigens termed ATLA, found by the serological study of ATL, are HTLV$_{MT-2}$ encoded polypeptides.

Key words: human leukemia virus/virus genome/cell free protein synthesis/anti-ATLA

Introduction

RNA tumor viruses have been isolated from various species of vertebrates such as birds and mammals and they have been implicated as natural oncogenic agents which may cause sarcomas, leukemias, malignant lymphomas and carcinomas (Aaronson and Stephenson, 1976; Hardy *et al.*, 1980). Evidence of an involvement of retroviruses in human diseases, however, was obtained only very recently.

Gallo and his co-workers were the first to isolate a type-C retrovirus from patients having cutaneous T-cell lymphoma, mycosis fungoides, and Sézary syndrome. This virus has been called human T-cell leukemia virus or HTLV (Poiesz *et al.* 1981; Kalyanaraman *et al.*, 1982). Later Hinuma and co-workers isolated HTLV from a cell line established from an adult T-cell leukemia patient (Hinuma *et al.*, 1981; Yoshida *et al.*, 1982).

Adult T-cell leukemia (ATL) was first described by Takatsuki and his associates as a new clinical form of T-cell malignancy (Takatsuki *et al.*, 1977; Uchiyama *et al.*, 1977; The T- and B-cell malignancy study group, 1981). ATL is the only known endemic form of human leukemia. It is found in southwest Japan where the occurrence of ATL correlates with formation of antibody to ATL-associated antigen or ATLA (Hinuma *et al.*, 1981). To analyze the gene products of HTLV origin and understand the etiological relationship between ATL and HTLV, we studied the genomes of HTLV and their genetic expression in cells as well as in a cell-free translation system.

HTLV$_{MT-2}$ shed from the MT-2 cell line that was established by co-cultivation of peripheral blood lymphocytes of an ATL patient with normal cord leukocytes (Miyoshi *et al.*, 1980), was used for this study. We found that the virion of HTLV$_{MT-2}$ contains mainly 24S RNA as a genome with discrete bands of 35S and 32S RNAs in small quantities. Also we demonstrated, for the first time, evidence that the proteins designated as ATLA are derived from and encoded in these genomic RNAs encapsidated in the HTLV virion.

Results

Genomic structure of HTLV$_{MT-2}$ virion RNA

The HTLV producer cell line MT-2 was labeled with [^{32}P]-phosphoric acid. ^{32}P-Labelled HTLV$_{MT-2}$ virion particles were harvested from the culture fluid as described in the experimental procedure. ^{32}P-Labeled virion RNA was then extracted by phenol-chloroform and poly(A)-containing RNA was selected by oligo(dT)-cellulose affinity chromatography. ^{32}P-Labelled RNA thus obtained from the HTLV$_{MT-2}$ virion particle was analysed by electrophoresis on 1% agarose gels under denaturing condition and the dried gel was autoradiographed to detect the ^{32}P-labeled RNA. As shown in Figure 1, lane 1, the HTLV$_{MT-2}$ virion particle contains predominantly 24S RNA with small amounts of 35S and 32S RNAs. The 24S RNA, which is smaller than RNAs of known defective retroviruses, sedimentated between 50 and 60S in sucrose density gradients under non-denaturing conditions, and was probably in the form of a dimer (data not shown). In addition to discrete bands of 35S, 32S and 24S RNA, we observed several bands smaller than 24S RNA. These smaller RNAs may represent degraded species since they were not found consistently in different preparations (data not shown).

To understand more precisely the genomic structure of HTLV$_{MT-2}$, we cloned the cDNA-HTLV$_{MT-2}$. cDNA-HTLV$_{MT-2}$ was prepared from HTLV$_{MT-2}$ virion RNA and cloned into vector pBR322 using the system described by Okayama and Berg (1982). pHTLV42 thus obtained contains a 2.2-kbp fragment which represents the 3′ end of the HTLV$_{MT-2}$ virion RNA. The fragment *Sma*I-*Taq*I corresponded to the U3R portion of the long-terminal repeat (LTR) of HTLV in which the sequence was identical to that reported previously (Seiki *et al.*, 1983). Using the *Sma*I-*Taq*I fragment we conducted RNA-DNA hybridization experiments. Cytoplasmic mRNA was prepared from MT-2 cells and RNAs in the gel were transferred to a nitrocellulose filter after electrophoresis as described by Thomas (1980). As shown in Figure 1, lane 2, the main band of mRNA which hybridized to the cDNA pHTLV42 *Sma*I-*Taq* fragment was 24S in size. Smaller amounts of 35S, 32S, 26S and 20S

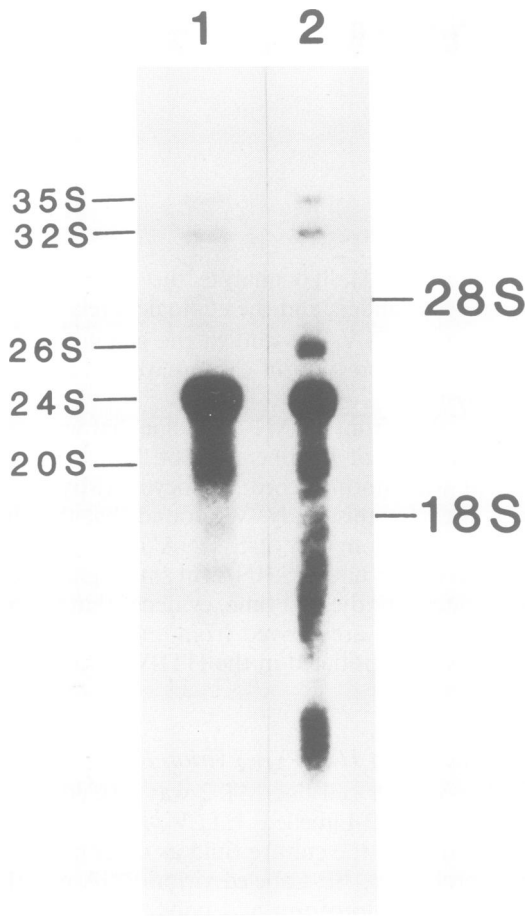

Fig. 1. Genomic structure of $HTLV_{MT-2}$. **(1)** ^{32}P-Labelled $HTLV_{MT-2}$ virion RNA analysed on agarose gel electrophoresis. Virion RNA was prepared as described in Materials and methods except that cells were incubated with [^{32}P]phosphoric acid (2 m Ci/ml) for 3 h. **(2)** Northern blotting of cytoplasmic mRNA from MT-2 hybridized to nick-translated pHTLV42 *Sma*I-*Taq*I fragment.

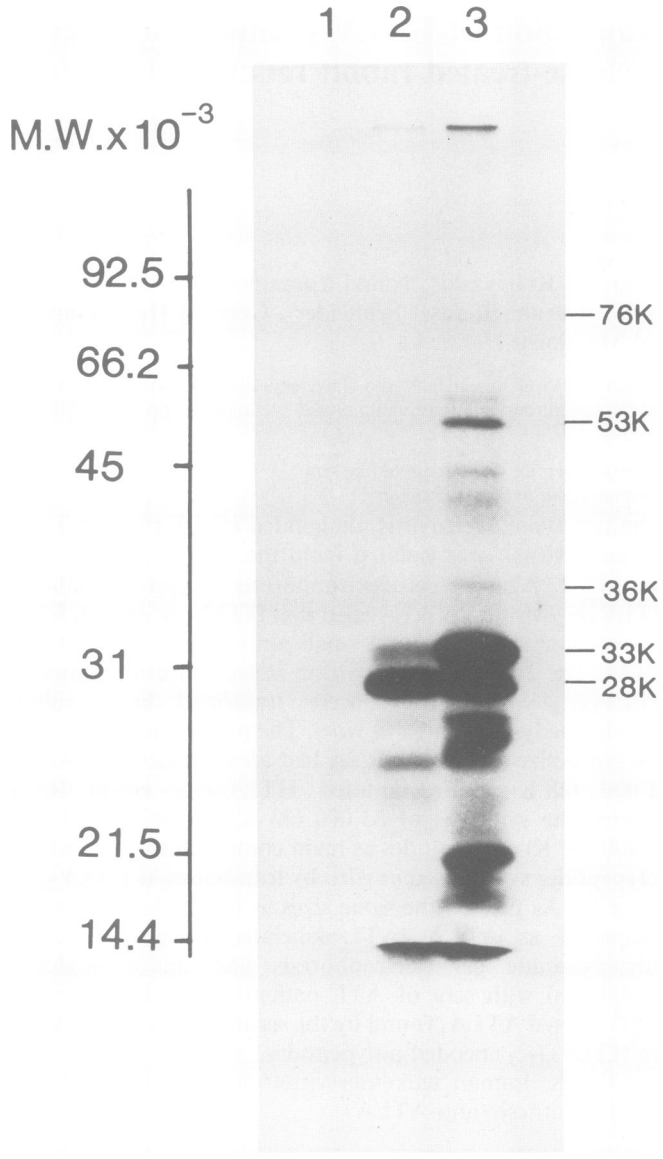

Fig. 2. Translation products of $HTLV_{MT-2}$ virion RNA in the rabbit reticulocyte lysate system. 24S $HTLV_{MT-2}$ virion RNA extracted from an agarose gel containing CH_3HgOH or unfractionated $HTLV_{MT-2}$ virion RNAs were incubated with [^3H]leucine in a rabbit reticulocyte lysate. The polypeptides synthesized were analysed by polyacrylamide SDS-electrophoresis on 12% gels. After electrophoresis fluorography was performed. **(1)** No mRNA, **(2)** 24S $HTLV_{MT-2}$ virion RNA, **(3)** unfractionated $HTLV_{MT-2}$ virion RNAs. Molecular standards used are phosphorylase B (92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), lysozyme (14 400).

mRNA were also detected. The profile of mRNAs specific to HTLV corresponds to those of $HTLV_{MT-2}$ virion RNAs except that the 26S and 20S mRNAs detected in the cytoplasm of MT-2 cells were missing in the virion RNAs. We also observed a smear of degraded RNA (smaller than 20S).

In vitro translational products of $HTLV_{MT-2}$ virion RNA

Since $HTLV_{MT-2}$ virion RNA consisted predominantly of 24S RNA with 35S and 32S as minor components and these RNAs bound to oligo(dT)-cellulose, we examined whether these $HTLV_{MT-2}$ virion RNAs may function as mRNA for the synthesis of virion polypeptides. The purified 24S RNA, as well as unfractionated RNAs of $HTLV_{MT-2}$, were used to program an *in vitro* protein synthesizing system, using a micrococcal nuclease-treated rabbit reticulocyte lysate (Pelham and Jackson, 1976).

$HTLV_{MT-2}$ virion RNA was purified and electrophoresed on a 1% agarose gel containing 7.5 mM methyl mercuric hydroxide and an RNA band corresponding to 24S in size was extracted by melting the gel with formamide. This purified 24S RNA and unfractionated $HTLV_{MT-2}$ virion RNAs were incubated with reticulocyte lysate in the presence of [^3H]leucine. Polypeptides synthesized in this reaction were then analysed by SDS-polyacrylamide gel electrophoresis. Figure 2 shows the results. When 24S RNA was used, a main band of mol. wt. 28 000 (28 K) was synthesized and small amounts of 33-K and 24-K polypeptides were also detectable (Figure 2, lane 2). The unfractionated RNAs directed the synthesis of polypeptides of mol. wts. 53 000 (53 K) and 33 000 (33 K), 26 000 (26 K), 25 000 (25 K), 21 000 (21 K), and minor components of 76 000 (76 K), 60 000 (60 K), 46 000 (46 K), 36 000 (36 K), in addition to the 28-K polypeptide (Figure 2, lane 3).

Immunoprecipitation of $HTLV_{MT-2}$ RNA gene products with anti-ATLA antibodies from ATL patients and antibodies raised specifically against ATLA components

Every ATL patient has specific antibodies which react with the antigens in ATL cell lines (Hinuma *et al.*, 1981;

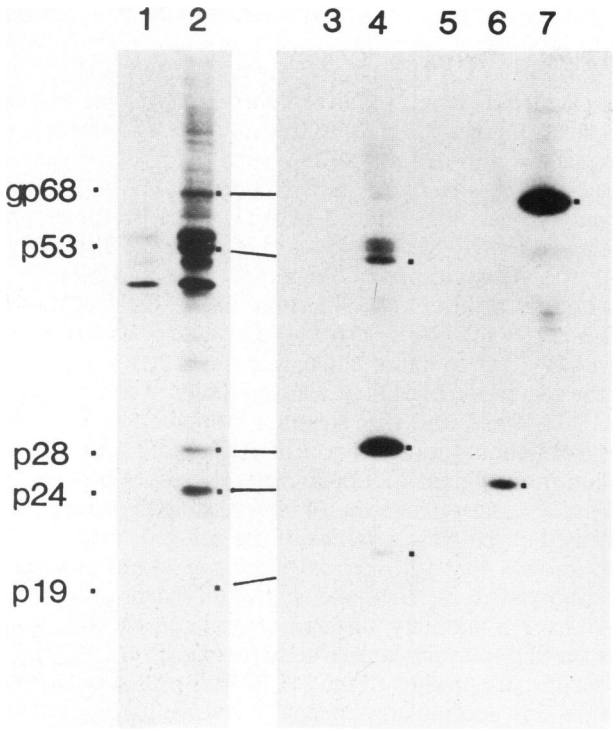

Fig. 3. Specificity of anti-ATLA antibodies. [^{35}S]Cysteine-labeled MT-2 cell extracts were immunoprecipitated. After incubating 4 h at 4°C, the immune complexes were precipitated using protein-A Sepharose. Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis. (1) Normal human serum, (2) serum from an ATL patient, (3) myeloma culture fluid, (4) mouse monoclonal antibody GIN-7, (5) normal rabbit serum, (6) anti-p24 antibody, (7) anti-gp68 antibody.

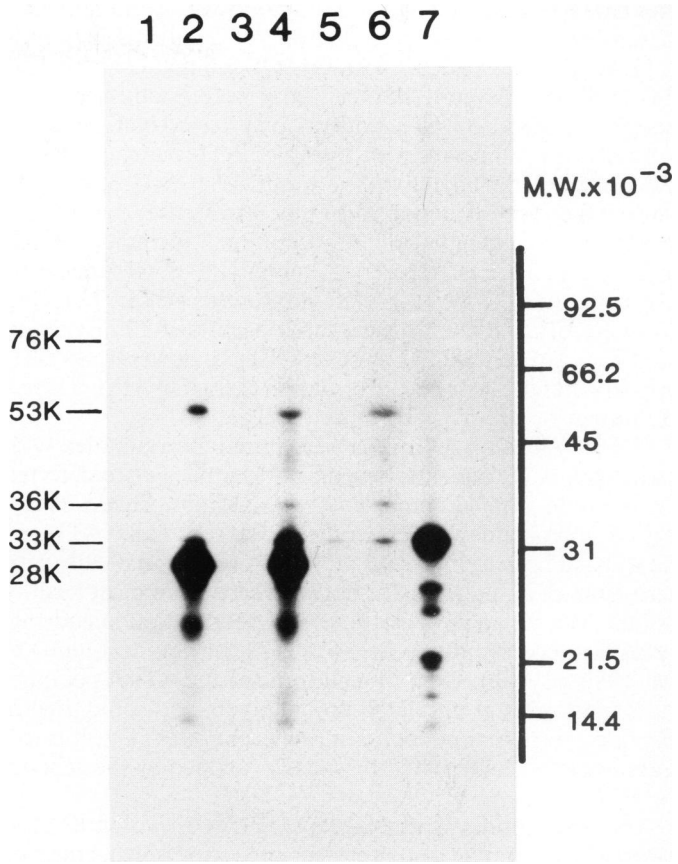

Fig. 4. Immunoprecipitation of polypeptides synthesized *in vitro* under the direction of $HTLV_{MT-2}$ virion RNAs with antibodies specific to ATLA. The *in vitro* translation product of unfractionated $HTLV_{MT-2}$ virion RNAs was prepared as described in Figure 3, lane 3 except that [^{35}S]methionine was used instead of [^{3}H]leucine. The samples were incubated with antibody specific to ATLA. The immune precipitates were analysed by SDS-polyacrylamide gel electrophoresis. (1) Myeloma culture fluid, (2) mouse monoclonal antibody, GIN-7, (3) normal human serum, (4) serum from an ATL patient, (5) normal rabbit serum, (6) anti-p24 antibody, (7) anti-gp68 antibody.

Yamamoto and Hinuma, 1982). If HTLV is closely related to ATL, it is also reasonable to postulate that ATLA polypeptides are encoded by HTLV. In this experiment, we examined whether the polypeptides synthesized under the direction of $HTLV_{MT-2}$ virion RNA were reactive with either anti-ATLA antibodies or antibodies specifically raised against the individual polypeptide components of ATLA (gp68, p28, p24). The specificity of these antibodies is shown in Figure 3. MT-2 cells labeled with [^{35}S]cysteine were disrupted and polypeptides reactive with these antibodies were immunoprecipitated and analysed by SDS-polyacrylamide gel electrophoresis. Anti-ATLA from serum of an ATL patient mainly reacts with gp68, p53, p36, p28, p24 and p19 (Figure 3, lane 2). These polypeptides were not recognized by normal human serum (Figure 3, lane 1). p76 and some other components of ATLA were scarcely detectable when the cell was metabolically labelled with [^{35}S]cysteine. The proteins of p76 and p53 are observed more clearly by labeling with other amino acids or by pulse-chase experiments (data not shown). A monoclonal antibody against p28 also recognized p53 and p19 (Figure 3, lane 4). Antibodies against p24 (Figure 3, lane 6) and against gp68 (Figure 3, lane 7) which were prepared in rabbits, as described in Materials and methods, recognize p24 and gp68, respectively, while normal rabbit serum recognizes neither of them (Figure 3, lane 5). Antibody against p24 also recognizes p53, when labeled with other amino acids (data not shown). Using these antibodies, the *in vitro* translation products of $HTLV_{MT-2}$ virion RNAs, such as those shown in Figure 2, lane 3, but labeled with [^{35}S]methionine, were immunoprecipitated. Figure 4 clearly demonstrates that almost every major product translated *in vitro* from $HTLV_{MT-2}$ virion RNAs immunoprecipitated with the sera related to ATLA and not with the control ones.

A monoclonal antibody GIN-7 clearly precipitates 53-K and 28-K polypeptides (Figure 4, lane 2), while control myeloma culture supernate recognizes no polypeptide (Figure 4, lane 1). Anti-ATLA serum from an ATL patient precipitates small amounts of the polypeptides 76 K, 36 K and 33 K along with those mentioned above (Figure 4, lane 4). Anti-p24 antibody precipitated 53-K and a small amount of 33-K polypeptides (Figure 4, lane 6). But the 33-K polypeptide recognized by anti-p24 is also precipitated by normal rabbit serum (Figure 4, lane 5).

Anti-gp68 antibody, however, precipitates a 33-K polypeptide that may be the non-glycosylated polypeptide of gp68 (Figure 4, lane 7). Several smaller polypeptides were also immunoprecipitated (Figure 4, lane 7).

Discussion

The new disease designated as ATL (adult T-cell leukemia) was first discovered in Japan (Takatsuki *et al.*, 1977; Uchiyama *et al.*, 1977). ATL is endemic in southwest Japan

and characterized by onset in adulthood with short survival. A serological study showed that all ATL patients had anti-ATLA, while no patients with other T-cell malignancies had this antibody. Several ATL cell lines were established from peripheral blood of ATL patients or after co-cultivation of normal cord leukocytes with T-cells of ATL patients. One of the cell lines designated MT-2 produces type-C retrovirus. Since it has been well established that retroviruses cause many kinds of malignant cell proliferation, including T-cell leukemia in various species of animals, it is reasonable to postulate that this newly isolated retrovirus (HTLV) derived from an ATL patient is the causative agent of ATL. Evidence for this postulate remains to be established, however. So far it appears that the genomic information carried by HTLV is not of human origin (Yoshida *et al.*, 1982).

When lysates of ATL cells were immunoprecipitated with sera from ATL patients, several polypeptides were detected (Yamamoto and Hinuma, 1982; Yamamoto *et al.*, 1982). Those polypeptides which reacted with sera from ATL patients have been termed ATLA. ATLA react specifically with sera from ATL patients but not with sera from most healthy adults. More than 20% of the healthy population in endemic areas also possess antibodies to ATLA, however (Hinuma *et al.*, 1982a; 1982b). Even though some of the ATLA polypeptides (p19, p24, glycosylated protein gp46, and some smaller peptides) were mainly found in the virion of HTLV, it is still questionable whether ATLA is really encoded by the genome of HTLV.

The polypeptides synthesized by $HTLV_{MT-2}$ virion RNA *in vitro* (Figure 2) may also represent precursor polypeptides of the HTLV genome. In the translation system, using retrovirus genomic RNAs, polypeptides synthesized *in vitro* represent precursors of several viral polypeptides (Jamjoom and Arlinshaus, 1978). The *gag* gene product of Rous sarcoma virus (RSV) is first synthesized as pr76 and then processed into smaller polypeptides in the cytoplasm; only the processed polypeptides are detected in virions (Vogt *et al.*, 1975; Shealy *et al.*, 1980). The precursor polypeptides of retroviruses are rapidly processed *in vivo* into smaller polypeptides. Some of these smaller polypeptides are the structural proteins of the virion. The precursor polypeptides are detectable in small quantities in the cell and are hardly demonstrable in virions. Thus, gp46, p24, p21, p19 and some other smaller polypeptides found mainly in virions and not detected in an *in vitro* translational system could be considered as the processed polypeptides, while p76, gp68 and p53 are precursor polypeptides since they are only found in cell extracts and/or in *in vitro* translational systems.

Recently, Seiki and co-workers reported the complete nucleotide sequence of the proviral gene of HTLV isolated from an ATL patient (Seiki *et al.*, 1983). They demonstrated that the HTLV provirus consists of 9032 bases within which exist possible *gag, pol* and *env* genes. The *gag* gene of HTLV may code for a polypeptide of 48 000 daltons (Pr 48^{gag}) which could be cleaved into three polypeptides. One of these polypeptides, which resides in the middle part of the *gag* gene, corresponds to p24 of ATLA and a sequence of 25 amino acids at the NH_2-terminal of this polypeptide was identical to such a sequence in p24 of HTLV (Manzari *et al.*, 1983). On the other hand, in the cells derived from an ATL patient, a protein of 53 000 daltons rather than 48 000 daltons was immunoprecipitated by anti-p24 antibody (Fgiure 3, lane 6). Manzari *et al.* (1983) demonstrated that one of the possible *gag* gene products of HTLV p19 resides in the NH_2 terminus of the *gag* gene. A monoclonal antibody GIN-7, which recognize p19 of ATLA, also recognizes p53 in ATL derived cells (Figure 3, lane 2). These data together with the data presented in Figure 2 of this study, which shows the cross reactivity of anti-p24 and GIN-7 with the 53-K polypeptide synthesized *in vitro* under the direction of $HTLV_{MT-2}$ RNA, strongly suggest that the p53 of ATLA is a precursor polypeptide of the *gag* gene product. The apparent difference in mol. wt. between p53 as reported here and pr48^{gag} as reported by Seiki *et al.* (1983) may reflect the fact that the HTLV proviral DNA isolated by Seiki and co-workers differs from MT-2 due to minor changes in the HTLV genome during the generation of HTLV. Our preliminary results indicate that HTLV p53 and p19 are phosphorylated *in vivo*. This post-translational modification of ATLA p53 and p19 may explain the apparent molecular size differences between p53 and pr48^{gag} and p19 and the 14 000 dalton polypeptide which is located at the NH_2 terminus of the *gag* gene product. It is well known that the retroviruses *gag* gene product is phosphorylated *in vivo* and phosphorylated polypeptides often have a mobility different from non-phosphorylated ones on SDS-polyacrylamide gels (Erikson *et al.*, 1977).

The primary product of the 24S RNA is p28. A polypeptide of this size accumulates in MT-2 cells in large quantities unlike other HTLV precursor polypeptides. If p28 is a precursor polypeptide it should be converted into smaller polypeptides soon after synthesis and the pool size of p28 in the cell should become small. Our pulse-chase experiments contradict this notion since there was little change in labeled p28. In contrast, labeled p53 was chased out completely when MT-2 cells were pulse-labeled and then chased for 2 h (data not shown). The possibility remains that p28 is a precursor polypeptide of p19 from the results using a mouse monoclonal antibody GIN-7. Some cell lines, however, contain p53 as well as p19, but not p28 (Tanaka *et al.*, 1983). Thus it may be more reasonable to suggest that p28 consists of a partial sequence of p19 and some unknown gene product. Such examples are already known in which a structural gene of a retrovirus is altered by incorporation of some other gene. The gene product is a fusion protein containing a portion of the structural gene product and the incorporated gene product, like the oncogene product (Lee *et al.*, 1980; Hanafusa *et al.*, 1980; Kawai *et al.*, 1980). The 33-K polypeptide, one of the major polypeptides synthesized *in vitro* by $HTLV_{MT-2}$ virion RNA, was immunoprecipitated by anti-gp68 antibody (Figure 4, lane 3). Using retrovirus virion RNA obtained from the virion, only *gag* gene-related products were synthesized in general. Recently, however, Pawson *et al.* (1980) reported that the *env*-related product was synthesized from virion RNA. Thus, the 33-K polypeptide may be an *env* gene-related product. The apparent difference of molecular size between gp68 and the 33-K polypeptide may simply reflect a difference in glycosylation. Further studies to investigate this possibility are now under way.

Materials and methods

Cells

The HTLV producer cell line MT-2 was maintained in RPMI-1640, supplemented with 10% fetal calf serum. MT-2 is a T-cell line which is >95% positive for ATLA. Labeling of MT-2 with [^{35}S]cysteine was performed as follows: 8 x 10^6 cells were incubated in 10 ml of medium containing 50 μCi/ml of [^{35}S]cysteine (Amersham, >600 Ci/mmol) for 16 h. The label-

ed cell suspension was washed three times with phosphate-buffered saline (PBS) and cells were lysed with lysis buffer (10 mM Tris-HCl, pH 8.0, 0.14 M NaCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40) at 4°C for 20 min (0.5 ml of lysis buffer/1 x 10^6 cell). Cell lysates were cleared by centrifugation at 14 000 *g* for 10 min at 4°C and used for immunoprecipitation.

Antisera

Sera from ATL patients and healthy adults were examined for their reactivity with ATLA by immunofluorescence. The titers of the sera selected for this study were 640 for serum from an ATL patient and <10 for serum from a healthy adult. Monoclonal antibody (GIN-7) raised against p28 of ATLA was obtained by standard hybridoma technology and a detailed characterisation of this monoclonal antibody has been described elsewhere (Tanaka *et al.*, 1983). Anti-p24 and anti-gp68 were prepared by injecting gel electrophoretically purified antigens into rabbits. Details of the preparation of these antibodies will be described elsewhere.

RNA extraction from purified virion

The culture fluid of MT-2 cells was harvested as follows. The culture fluid was first centrifuged at 400 *g* for 4 min to remove the cells and the supernatant was centrifuged again at 5000 r.p.m. for 10 min to remove cellular debris. The supernatant was then mixed with 10 x NTE (NTE: 0.1 M NaCl; 10 mM Tris-HCl, pH 7.4; 1 mM EDTA) to make a final concentration of 3 x NTE. This mixture was then centrifuged at 30 000 r.p.m. for 60 min in a Beckman type 35 rotor. The pelleted virion particles were then suspended in 25 ml of 3 x NTE and layered on the top of a sucrose density gradient [13 ml, 15–60% (w/v) in NTE] in a Beckman SW 27 rotor. The sample was centrifuged at 25 000 r.p.m. at 4°C for 60 min. After centrifugation the virion fraction was removed and mixed with an equal volume of RNA extraction buffer (2% SDS, 1 mg/ml pronase K, 6 x NTE). This mixture was incubated at 37°C for 1 h and viral RNAs were extracted with phenol/chloroform (3 x NTE saturated phenol: chloroform = 1:1). Poly(A)-containing mRNA was selected by sequential passing of the sample through an oligo(dT)-cellulose column (Aviv and Leder, 1972).

Cellular mRNA and RNA-DNA hybridization

Cellular mRNA was prepared from MT-2 using guanidium thiocyanate as described by Chirgwin *et al.* (1979). Cellular mRNAs were selected by oligo(dT)-cellulose and 0.1 μg of mRNA was separated by agarose gel electrophoresis. After electrophoresis, RNAs on the gel were transferred to a nitrocellulose filter and RNA-DNA hybridization was performed as described by Thomas (1980). About 1 x 10^7 c.p.m. (4 x 10^8 d.p.m./μg DNA) of a nick-translated [^{32}P]probe, prepared with a nick translation kit (Amersham), was used for the hybridization.

Cell-free protein synthesis

A rabbit reticulocyte lysate, treated with micrococcal nuclease, was purchased from Amersham. The reaction was carried out at 30°C for 60 min in the presence of either [^{3}H]leucine (Amersham 130–190 Ci/mmol) or [^{35}S]-methionine (Amersham >600 Ci/mmol). Polypeptides synthesized under the direction of added RNA were analysed on SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970) followed by autoradiofluorography (Bonner and Laskey, 1974).

Immunoprecipitation assay

Polypeptides synthesized *in vitro* under the direction of $HTLV_{MT-2}$ virion RNA were diluted 4 times with low extraction buffer and mixed with 5 μl sera and incubated at 4°C for 16 h. Immune complexes were recovered using 10 mg of protein A-Sepharose (Pharmacia) as described by Kessler (1975). Immunoprecipitation of MT-2 cell extract was performed as described previously (Yamamoto and Hinuma, 1982).

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Note added in proof

We changed the name of the virus from ATLV to HTLV based on the article by Yoshida *et al. Science (Wash.)*, (1983) **222**, 1178.