JOURNAL OF VIROLOGY, May 1986, p. 508–512
0022-538X/86/050508-05$02.00/0

Vol. 58, No. 2

Frequent Partial Deletion of Human Adult T-Cell Leukemia Virus Type I Proviruses in Experimental Transmission: Pattern and Possible Implication

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Human T-cell leukemia virus type I (HTLV I) propagated in human diploid fibroblast IMR90 was transmitted to human promyelocytic leukemia HL60 cells by coculture. Of 14 provirus-positive HL60 clones, five harbored only defective proviruses, five had defective proviruses in addition to full-sized HTLV I, and four had full-sized proviruses integrated in their chromosomes. The frequency of defective proviruses was unexpectedly high (41% of total proviruses). Analysis of the genomic structure of these defective proviruses revealed polarity of deletion, that is, preferred conservation of the 3′ end of the proviral genome (*pX* and the 3′ long terminal repeat). The implication of these findings are discussed with reference to the replication and pathogenesis of HTLV I.

Defective human T-cell leukemia virus type I (HTLV I) proviruses are frequently observed in tumor cells of adult T-cell leukemia (ATL) patients (17, 19). In other retroviruses, such as Moloney murine leukemia virus, the formation of partially deleted proviruses is known (14). In the case of avian leukosis virus, promoter insertion activation of cellular oncogene was frequently associated with the partial deletion of proviral genomes (4). Thus, deletion of the viral genome seems to be a fairly commonplace occurrence in the replication of certain retroviruses. However, the details of the pattern and mechanism of deletion have not been fully resolved. HTLV I is best suited for solving this problem since there is no related endogenous virus in mammalian cells. We recently reported the isolation of HTLV I from peripheral blood lymphocytes (PBL) of ATL patients by in vitro coculture with the human diploid fibroblast IMR90 (20). IMR90 was shown to be permissive for the replication of HTLV I (K. Hiramatsu and H. Yoshikura, Int. J. Cancer, in press). Other investigators have detected HTLV I genome in various cells cocultured with ATL cells (5, 9), but only a few of them were permissive for replication of the virus (1). In this study we utilized the HTLV I-infected IMR90 cells to transmit HTLV I into human promyelocytic leukemia HL60 cells. The proviral structure integrated in HL60 cells was analyzed by Southern blot hybridization.

MATERIALS AND METHODS

Preparation of PBL from an ATL patient. PBL cells were purified by centrifugation through lymphocyte separation medium (Litton Bionetics) and washed several times with saline.

Cells. IMR90 cells (10) in the passage levels of population doubling level 23 to 30 were obtained from T. Kuroki, Institute of Medical Science, University of Tokyo. HL60 cells (3) were provided by M. Terada, National Cancer Research Institute, Tsukiji, Tokyo. The culture medium used was Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum. All of the cultures were carried out in a humidified CO_2 incubator at 37°C.

Probes. HTLV I proviral DNA p23 (2) and pATK32 (12) were provided by M. Yoshida, Cancer Research Institute, Tokyo, Japan. Partial genome probes U_5, *gag*, *env*, *pX*, and U_3 were made by restricting p23 DNA with *Sst*I-*Sma*I, *Sma*I, *Xho*I, *Cla*I-*Sma*I, and *Sma*I-*Sst*I, respectively.

Hybridization. Cellular DNA (3 to 10 μg per lane) was cleaved with restriction enzymes, phenol extracted, and ethanol precipitated. Digested DNA was run in 0.8% agarose gel and transferred to nitrocellulose membrane (15). The membrane was hybridized with nick-translated probes (10^8 cpm/μg) and washed twice in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min at 65°C.

RESULTS

Structure of HTLV I provirus integrated in the tumor cells of an ATL patient. A Southern blot analysis of HTLV I provirus in the cellular DNA obtained from fresh PBL of an ATL patient designated patient A1 (a 49-year-old male hospitalized in the Third Department of Internal Medicine, University of Tokyo) is shown in Fig. 1A. This HTLV I isolate had a unique *Sst*I site in the long terminal repeat (LTR; Fig. 1B). The *Pst*I site at position 7268 (shown in parenthesis) is present in λATK-1 but was absent in the A1 isolate. *Eco*RI had no cutting site inside the proviral genome. *Eco*RI-digested DNA revealed a single band detectable with HTLV I probe, and *Pst*I digest showed two cell-virus junction fragments. Hence, it is clear that ATL cells of this patient had only one copy of HTLV I provirus. Digestion with *Sst*I showed a band of 8.4 kilobases (kb), which is the reported full-length size of an intact HTLV I provirus (2), suggesting that this provirus had an intact genome.

Establishment of HL60 clones transmitted with HTLV I. The HTLV I propagated in IMR90 cells (20) was transmitted to HL60 cells. The method of transmission was as follows. HL60 cells adhered to the monolayer of HTLV I-infected IMR90 cells but not to the control IMR90 cells, which were not infected with HTLV I (Hiramatsu and Yoskikura, in press). The medium was changed every 3 to 4 days. Less firmly adherent cells were removed by vigorous shaking of the culture bottle. During 3 months of culturing in this manner, IMR90 cells gradually died due to senescence (10). The remaining cells were collected and subjected to cloning

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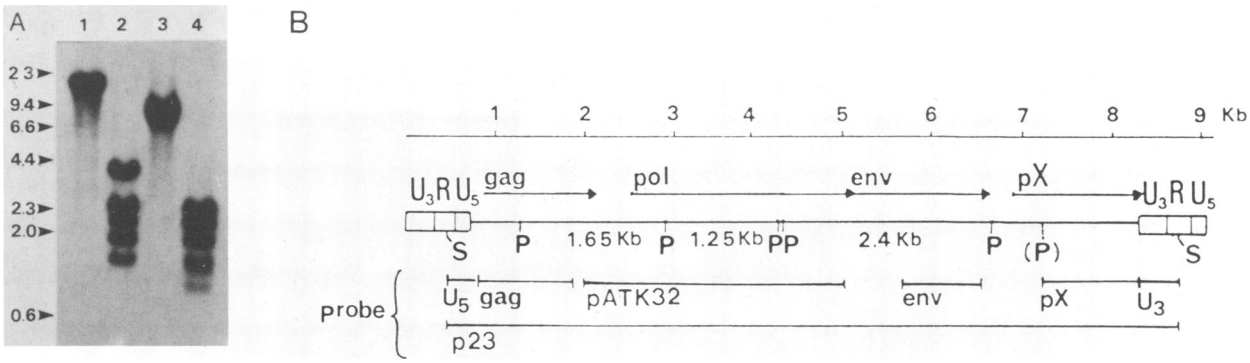


FIG. 1. Genomic structure of the HTLV I provirus integrated in the PBL obtained from ATL patient A1. (A) Nitrocellulose blots of digests of DNA from purified PBL cells. Lanes: 1, *Eco*RI digests; 2, *Pst*I digests; 3, *Sst*I digests; 4, *Sst*I-*Pst*I digests. All digests were hybridized with probe p23. Molecular marker sizes are shown in kb. (B) Restriction map of the HTLV I isolate of patient A1 in comparison with λATK-1 (12), showing all of the HTLV I probes used. In the A1 isolate there was an additional unique *Sst*I site in the R region of the LTR which is absent in λATK-1. The *Pst*I site at position 7268 of λATK-1 (shown in parenthesis) was absent in the A1 isolate.

by limiting dilution. We obtained 48 clones, 14 of which were positive for HTLV I proviruses.

High frequency of defective proviruses. A Southern blot analysis of the 14 clones mentioned above is shown in Fig. 2A. An *Sst*I digest of cellular DNA of these clones was blotted and hybridized with HTLV I whole-genome probe p23. The arrows show bands smaller or larger than the 8.4-kb band (the size of a complete HTLV I provirus). The bands smaller than 8.4 kb should represent defective proviruses (in this hybridization condition, the cell-virus junction fragments were not detectable). Band c, however, which is much larger than 8.4 kb, was found to be a defective provirus which lacked part of the 5′ LTR, including the *Sst*I site. Nine defective proviruses were detected in this manner.

Some of the HL60 clones with single integrated proviruses which were analyzed by double digestion of cellular DNA with *Sst*I and *Pst*I, followed by hybridization with p23 probe, are shown in Fig. 2B. By this digestion procedure, five internal fragments of 2.4, 2.1, 1.65, 1.25, and 1.0 kb are expected from a complete proviral genome (e.g., lane 4 in Fig. 1A). Clone 6 lacked 2.4- and 2.1-kb bands. Clone 23 lacked 2.4-, 1.65-, 1.25-, and 1.0-kb bands. Clone 30 lacked 2.4- and 2.1-kb bands. Clone 3C lacked 1.65-, 1.25-, and 1.0-kb bands. In addition to the absence of some of the internal bands, these clones also had extra bands of different sizes (indicated by arrows). Clone 6 lacked two expected internal bands but had an extra band of 4.4 kb which was almost equivalent to the sum of the two lacking bands. This band was shown to be hybridizable with both *pX* and *env* probes (data not shown). Therefore, clone 6 must have a very small deletion or a point mutation involving the *Pst*I site at position 6729. Clone 1C was initially considered to have a complete genome size by analysis with *Sst*I digest, but the second largest band in lane 5 showed a size slightly smaller than 2.1 kb, indicating a small deletion of less than 0.1 kb in the *pX*–3′-LTR region. Clone 3C also had a deletion of less than 0.1 kb in the *pX*–3′-LTR region. A total of at least 11 of 27 proviruses (41%) were found to be defective. The total number of proviruses was estimated by Southern hybridization of *Eco*RI-digested cellular DNA of HL60 clones (Hiramatsu and Yoshikura, in press).

Preferred conservation of *pX* and 3′ LTR in defective proviruses. To localize the deletion of the HTLV I proviral genome, six identical agarose gels of *Sst*I-digested DNA were run, blotted, and hybridized with six different HTLV I partial genome probes (see Fig. 1B for all of the HTLV I probes used in this study) (Fig. 2, C to H). All of the defective provirus bands were detected by *pX* probe (arrows in Fig. 2E), but some of them were not detected by other partial genome probes such as *gag*, pATK32, or *env*. The U_3 probe also detected all the defective bands (arrows in Fig. 2G), providing evidence that the U_3 portion of the 3′ LTR is constantly conserved. The U_5 probe, however, did not detect the provirus h6d (arrow c in Fig. 2A and E, arrow in lane 6 of Fig. 2G). This indicates that the provirus h6d lacks the U_5 portion of the 5′ LTR. Also, it should be noted that clone 23 contains only a single defective provirus and has a cell-virus junction fragment that is very weakly hybridizable with the U_3 probe (asterisk in Fig. 2G). This indicates that provirus 23 lacks most of the U_3 portion of the 5′ LTR. Other proviruses seemed to retain intact 3′ and 5′ LTRs, judging from the number and hybridization intensity of cell-virus junction fragments detected by the U_3 and U_5 probes (Fig. 2G and H). The pattern of deletion of the defective proviruses analyzed above is illustrated in Fig. 3.

Rare deletions after establishment of proviral integration. To see whether these deletions occurred before or after integration, we recloned clones 9 and 1C, obtaining a total of 16 subclones. The occurrence of deletion was high (more than 40% of total proviruses). Hence, if deletion occurred after integration, we should have been able to detect some deleted proviruses among these subclones. The results are shown in Fig. 4. The size and number of integrated provirus were identical among the parent and sister clones. It is clear from this result that deletion never or very rarely occurs after the establishment of proviral integration.

DISCUSSION

Our analysis of the defective proviruses indicated that there was a characteristic pattern of deletion. First, 3′ LTR is conserved in every defective provirus, whereas partial deletion of 5′ LTR is not infrequent. Second, the *pX* region is well conserved despite the frequent deletion of other structural genes such as *gag*, *pol*, and *env*. With regard to the former point, it is interesting that some ATL patient cells contain proviruses which lack one of the two LTRs (17, 18). Yoshida et al. (18) have cloned a provirus from the primary tumor cells of an ATL patient lacking the U_5 portion of the 5′ LTR. This clone is similar to our defective provirus h6d (type 8). This preferential deletion of 5′ LTR is also reported in avian leukosis virus-transformed tumors (4) and in the structural analysis of chicken endogenous proviruses (6).

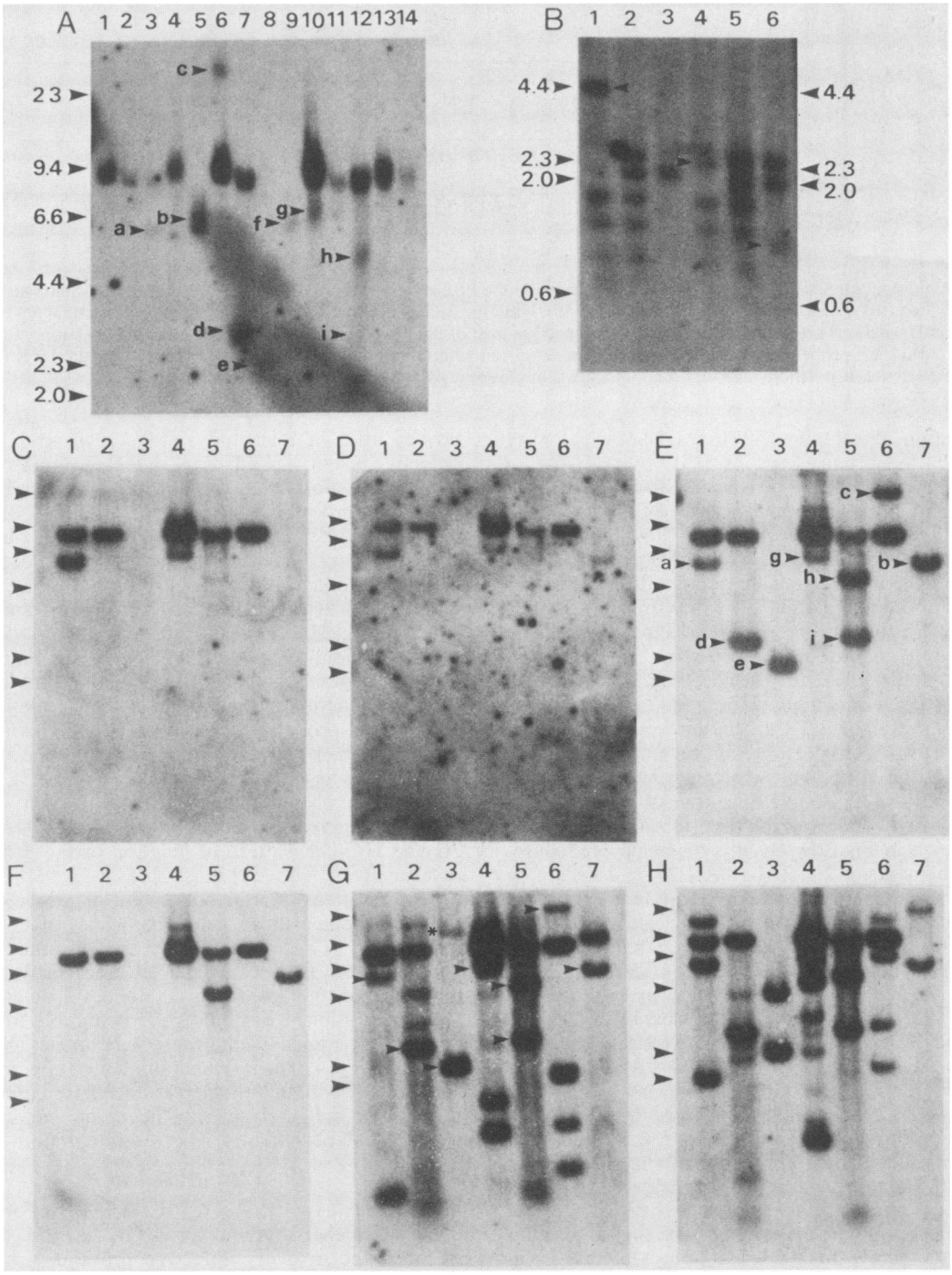


FIG. 2. Southern blot analysis of the defective HTLV I proviruses integrated in HL60 clones. (A) *Sst*I cellular DNA digests hybridized with p23 probe. Lanes 1 to 14 represent clones 6, 9, 10, 1C, 3C, h, 21, 23, 30, 33, 35, 39, 41, and 42, respectively. Defective proviruses (arrows) were as follows: a to i represent proviruses 10d, 3C, h6d, 21d, 23, 30, 33d, 39d-1, and 39d-2, respectively. (B) Cellular DNA of clones with single integrated provirus doubly digested by *Sst*I and *Pst*I and hybridized with p23 probe. Lanes 1 to 6 represent clones 6, 9, 23, 30, 1C, and 3C, respectively. Arrows indicate bands of different sizes from those of expected internal fragments of a full-size provirus. (C to H) Six identical filters of *Sst*I-digested cellular DNA hybridized with six different subgenomic probes. The probes were *gag*, pATK32, *pX*, *env*, U_3, and U_5, respectively. Lanes 1 to 7 represent clones 10, 21, 23, 33, 39, h, and 3C, respectively. All of the defective proviruses were detectable with *pX* and U_3 probes (arrows in E and G; see text for further description). Molecular size markers are 23, 9.4, 6.6, 4.4, 2.3, and 2.0 kb from the top.

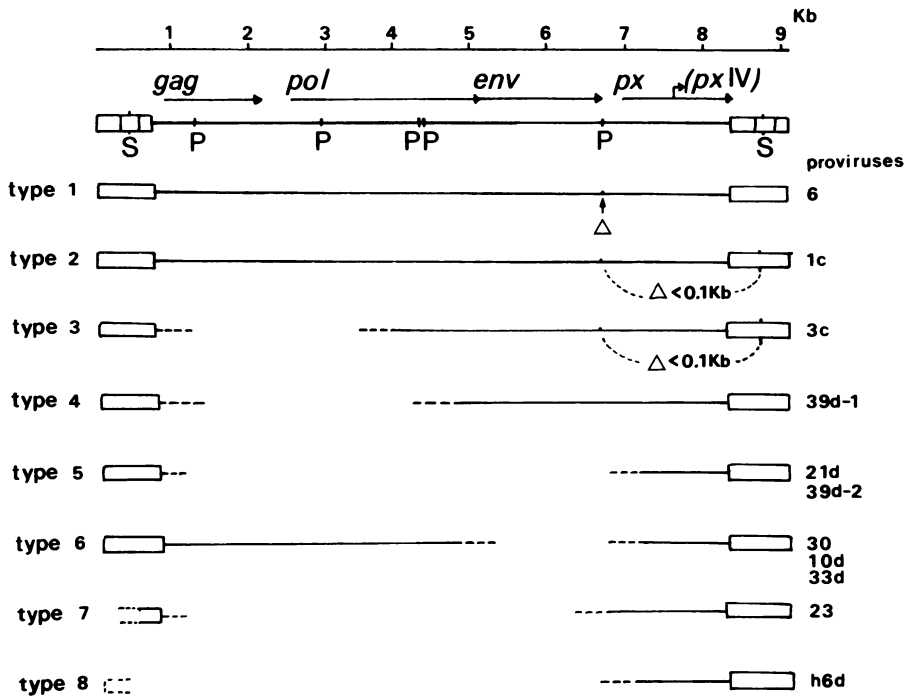
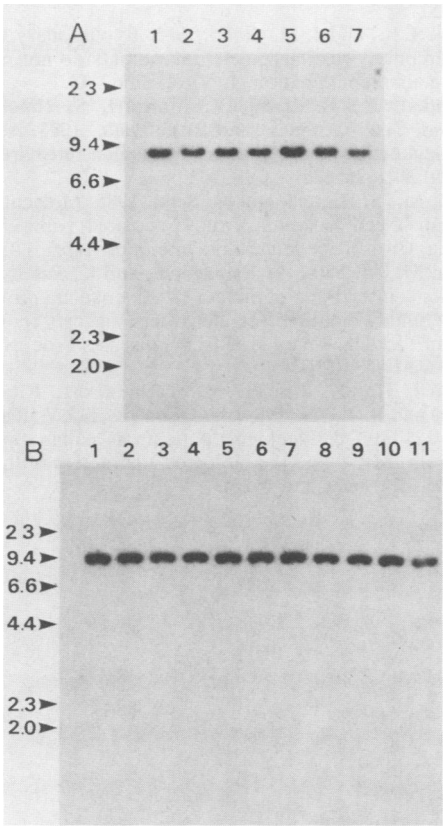


FIG. 3. Eight types of deletion (Δ) in defective HTLV I proviruses. Solid lines indicate the conserved proviral genome. Broken lines indicate ambiguous deletion boundaries. Type 1 has either a small deletion or mutation involving the *Pst*I site. Types 2 and 3 have small deletions (<0.1 kb) between the *Pst*I and *Sst*I sites. Type 7 retains the U_5 portion of the 5′ -LTR region, but most of the U_3 portion is deleted. Type 8 lacks the U_5 portion of the 5′ LTR (see text for further description).



Thus, there appears to be a polarity in the deletion pattern for certain retroviruses; that is, the 5′ LTR side is more prone to deletion than the 3′ LTR side.

Several factors have been assumed to cause proviral deletions in retrovirus infection. In the case of avian leukosis virus-transformed tumors, two factors were considered to be at work: (i) the selective pressure of host immune surveillance and (ii) the higher proliferation rate of transformed clones having defective proviral genomes (4). In our study, these selective pressures were not at work because it was an in vitro study and the doubling time of the cells with defective or complete proviral genomes did not differ (data not shown).

It has been reported that a defective provirus was produced from subgenomic mRNA through the reverse transcription and reintegration process (7). This mechanism does not seem to be appropriate in our case for the following reasons. First, in the case of HTLV I, a splice donor site is situated in the R region of 5′ LTR (13), so the subgenomic mRNA is devoid of a tRNA binding site, which is required for the initiation of reverse transcription. Second, most of our defective proviruses did not have structural similarity to the spliced subgenomic mRNA. An alternative mechanism to produce deletion after the establishment of proviral integration is the spontaneous deletion of the proviruses. This mechanism also seems unlikely since a subcloned population

FIG. 4. Deletion never or very rarely occurs after the establishment of integration of proviruses. (A) Cellular DNA *Sst*I digests hybridized with probe p23. Lanes: 1, clone 1C; 2 to 7, subclones of clone 1C. (B) Cellular DNA *Eco*RI digests hybridized with probe p23. Lanes: 1 to 10, subclones of clone 9; 11, clone 9.

of HTLV I-infected HL60 cells did not shown any alteration of their proviral genomes (Fig. 4). It therefore seems likely that the observed defective proviruses were produced during some steps in the replication cycle of the retrovirus before the establishment of integration, namely, (i) reverse transcription, such as in the copy choice mechanism (8, 16) of the virion RNA, (ii) aberrant cleavage of circular proviral DNA by the viral endonuclease (11), and (iii) the integration step itself. We cannot conclude what is the real cause of deletion at work in our case, but the model of the deletion mechanism should explain the occurrence of the polarity of the observed defective proviruses. The deleted HTLV I proviruses identified in the cloned HL60 cells may not have been produced in HL60 cells themselves, but could have been produced by integration of the defective viruses which were present in IMR90 cells and rescued by the replication-competent helper.

Since the *pX* product has been implicated in the transformation of human T cells (18), the exceptionally high probability of conservation of the *pX* region kindles our teleological speculation of the pathogenesis of HTLV I. Because of the preferred conservation of the 3′ end of the proviral genome, the *pX* region has an increased probability of conservation and expression of gene product. Actually, the *pX* region is reported to be conserved in all tumor cells of ATL patients (18). One example, mentioned above, is the provirus isolated by Yoshida et al. (18). It is defective in all regions except for 5′ U_3, *pX*, and 3′ LTR. It is important to determine whether this type of defective clone is transcriptionally active or not. Clone 23 (type 7) with a defective 5′ U_3 was shown to be inactive in transcription (unpublished data). Other proviruses, such as types 5 and 8, are under investigation.

ACKNOWLEDGMENT

We thank M. Kato for technical assistance, M. Yoshida for his kind present of the HTLV I probes, and Paul Cox for reviewing this manuscript.

This work was supported by grants-in-aid from the Ministry of Education and the Ministry of Health and Welfare.

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