Epigenetic Control and Reintegration of Extrachromosomal Proviral DNA in HL60 Cells Chronically Infected with Human T Cell Leukaemia Virus Type 1

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(Accepted 30 November 1987)

SUMMARY

Extrachromosomal human T cell leukaemia virus type 1 (HTLV-I) proviruses are persistently maintained in HTLV-I-infected human promyelocytic leukaemia (HL60) cells even 24 months after viral infection. By successive recloning of these HTLV-I-infected clones, and by Southern blot analysis of their HTLV-I proviruses, we concluded the following. The copy number of extrachromosomal proviruses fluctuated, and this fluctuation was probably dependent on the epigenetic conditions in the host cell, HL60. The transient appearance of a high copy number of extrachromosomal proviruses was followed by an increase in the copy number of integrated proviruses. Persistence of extrachromosomal proviruses appeared to be caused by an intracellular rather than an intercellular mechanism.

INTRODUCTION

In several virus transmission experiments in vitro with different host cell–retrovirus combinations, persistence of extrachromosomal proviruses has been noticed even long after the initial viral transmission (Guntaka et al., 1976; Varmus & Shank, 1976; Ringold et al., 1978). These proviruses are considered to be generated by reverse transcription of template viral RNA (Varmus & Shank, 1976). However, little is known about the regulation and biological significance of these provirus molecules. During our study on the mode of human T cell leukaemia virus type 1 (HTLV-I) transmission using HL60, a human promyelocytic leukaemia cell line (Collins et al., 1978), we found that extrachromosomal provirus molecules were maintained even 24 months after virus infection and integration (Hiramatsu et al., 1986). The copy number of the extrachromosomal proviruses of HL60 cells varied from 0.01 to 0.1 per cell. We recloned these HTLV-I-infected HL60 clones, and analysed extrachromosomal and integrated proviruses by Southern blot hybridization. An unexpected fluctuation in the number of extrachromosomal proviruses and the reintegration capability of these molecules is reported. The possible mechanism and biological significance of these observations are discussed.

METHODS

Cells. Infection and derivation of HL60 clones have been described elsewhere (Hiramatsu et al., 1986). Briefly, these clones were obtained by limiting dilution of HL60 cells which had been cocultured for 3 months with virus-infected human diploid fibroblasts (IMR90) (Yoshikura et al., 1984). Fourteen of 48 clones contained integrated HTLV-I proviruses (Hiramatsu & Yoshikura, 1986). All cell cultures were grown in Dulbecco’s MEM supplemented with 5% foetal calf serum at 37°C with 5% CO2. Cells were passaged every 2 days by transferring one third of the suspended culture to fresh medium.

Recloning of HTLV-I-infected HL60 clones. The frozen stock cells, which had been cultured for 3 to 5 weeks after initial cloning, were thawed and recloned by the limiting dilution method.
Fig. 1. Forty-eight HL60 clones were obtained by limiting dilution after coculture with HTLV-I-infected IMR90 cells and divided into six groups according to their provirus copy number. The number of members in each group was then plotted against their integrated provirus copy number. Copy number was estimated by analysing EcoRI-digested cellular DNA with a full-length HTLV-I probe by Southern hybridization.

**RESULTS**

**Non-Poisson distribution of provirus copy number among HTLV-I-infected HL60 cells**

HL60 clones obtained after coculture with HTLV-I-infected IMR90 cells were grouped according to the copy number of the integrated proviruses in their genomes and plotted as shown (Fig. 1). The pattern of distribution did not conform to that of Poisson [$\chi^2 = 4.74 > \chi^2(0.05) = 3.84, 1$ degree of freedom]. This contradicted the assumption that every virus integration event was an independent event. It was suspected, therefore, that the proviruses in some of the above clones might have a tendency to increase after the initial integration. The clones could be divided into two groups by the presence or absence of persistent extrachromosomal proviruses (Table 1) (Hiramatsu et al., 1986). It was noted that most of the clones with multiple integrated proviruses were those with persistent extrachromosomal proviruses.

**Amplification of integrated provirus copies in HTLV-I-infected HL60 cells**

We suspected that some copies of integrated proviruses might represent recent integration events occurring after the initial infection/integration event. To prove this hypothesis, we recloned some of the above clones, and the copy number of integrated proviruses of each
Fig. 2. Southern blot analyses of HTLV-I-infected HL60 clones. (a to d) EcoRI-digested total cellular DNA of subclones and parent clone analysed with a full-length HTLV-I genomic probe. (a) Lanes 1 to 9, subclones; lane 10, parental clone 33. (b) Lane 1, parental clone 35; lanes 2 to 14, subclones. (c) Lane 1, parental clone 21; lanes 3 to 6, subclones. (d) Subclones of clone h. (e to g) SacI-digested total cellular DNA of parent and subclones of clone h was probed by whole genomic (e), U3 (f) and U5 (g) probes, respectively. Lanes 1 of (e) to (g), parent clone h; lanes 2 to 5 of (e) to (g), subclones of clone h. The subclones of h analysed in lanes 2 to 5 correspond to those in lanes 1 to 4 of (d). The arrows indicate proviruses that are common to all subclones. Positions of size markers (HindIII fragments of wild-type λ DNA) are denoted to the left of each gel. They are 23 kb, 9.4 kb, 6.6 kb, 4.4 kb, 2.3 kb and 2.0 kb from the top downward. These are common to all subsequent figures except that only the top four or five markers are shown in some gels.

Table 1. Integrated and extrachromosomal proviruses of 14 clones of HTLV-I-infected HL60 cells

<table>
<thead>
<tr>
<th>Copy number</th>
<th>Names of clones*</th>
<th>Number of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6, 9, 23, 30, 35, 41, 1C, 3C</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>10, 42</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>33, 3h</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>21, 39</td>
<td>2</td>
</tr>
</tbody>
</table>

* Underlined clones contain extrachromosomal proviruses.

subclone was estimated by Southern blot hybridization (Fig. 2) after EcoRI digestion which does not cut within the HTLV-I genome (Hiramatsu et al., 1987).

Fig 2(a) shows the Southern hybridization patterns of the subclones of clone 33; heterogeneity in the copy number of proviruses was observed. It was noted, however, that there were two
proviral copies shared by all the subclones and the parent clone (arrows; Fig. 2a) indicating that all subclones were derived from a single ancestral clone with two integrated proviruses. The same result was obtained by analysing subclones of other clones (Fig. 2b to d). In the case of clone h, the identity of three copies of proviruses shared among subclones was further demonstrated by analysing SacI-digested cellular DNA. This enzyme has a single recognition site within the long terminal repeat (LTR) of the HTLV-I genome (Hiramatsu et al., 1987). By hybridizing the blot with LTR (U3 or U5) probes as well as a full length HTLV-I probe, the cell–virus junction fragments of each integrated provirus as well as the 8.4 kb HTLV-I genomic fragment were visualized (Fig. 2e to g). The migration pattern of the cell–virus junction fragments (arrows in Fig. 2f and g) supported the presence of three shared copies of proviruses. It was concluded, therefore, that the copy number of proviruses in some of the clones did increase during subsequent culture.

To determine whether provirus copies continued to increase or not, we recloned subclone h-3 3 weeks after the initial subcloning. Total cellular DNA was extracted from nine of these subclones 3 weeks after recloning by limiting dilution. There was no detectable increase of copy number (Fig. 3a), indicating that the copy number increase was not a steady state occurrence.

Spontaneous and transient increase of extrachromosomal proviruses in HTLV-I-infected HL60 cells

Fig. 3(b to d) shows Southern blots of EcoRI-digested total cellular DNA of new clones of subclones 35-2 (lane 3 of Fig. 2b), 35-3 (lane 4 of Fig. 2b) and 35-15 (lane 14 of Fig. 2b). A high copy number of extrachromosomal proviruses appeared in some subclones (lanes 5, 6, 8, 9, 12 to 16 of Fig. 3b; lanes 1, 3 to 7, 11, 12 of Fig. 3c; and lanes 3, 5, 6, 9 to 11 of Fig. 3d). For example, closed circular forms with one as well as two LTRs could be observed (Fig. 3d, lane 11 large arrow, linear provirus; small arrow, closed circular forms). It should be noted that, in the previous Southern blots in Fig. 2, extrachromosomal proviruses did exist but were not observed due to a copy number of less than 0.1 per cell. Some extrachromosomal proviruses exceeded 10 copies per cell (e.g. lane 11 of Fig. 3d). Interestingly, there were no new integrated proviruses except for those observed in lanes 13 and 14 (Fig. 3c). The frequency of the copy number increase of integrated proviruses, therefore, was just as low as with clone h-3 (Fig. 3a).

We also tested whether this high copy number of extrachromosomal proviruses in the clones shown in Fig. 3 was stably maintained during subsequent culture (Fig. 3e). The result indicated that extrachromosomal DNA disappeared from all the clones after 2 weeks of continuous culture. The copy number of the extrachromosomal proviruses of the clone 35-15-11 decreased to 0.1 per cell (data not shown). From this result it was concluded that the spontaneous increase of extrachromosomal proviruses was transient in nature.

Reintegration of extrachromosomal proviruses

To know the consequence of the spontaneous increase of the extrachromosomal proviruses described above, we recloned two subclones of 35-15. Subclones 35-15-11 (with a high extrachromosomal HTLV-I DNA copy number; lane 11 of Fig. 3d) and 35-15-14 (with undetectatable extrachromosomal HTLV-I DNA; lane 14 of Fig. 3d) were recloned and the copy number of integrated proviruses was estimated (Fig. 4a, b). It was found that the frequency of increase in copy number of integrated proviruses was significantly higher in 35-15-11 (50%) compared to 35-15-14 (20%). No visible extrachromosomal HTLV-I proviruses were observed. Clone 35-14-11 was again subjected to recloning after its loss of high copy number extrachromosomal HTLV-I DNA. Resultant subclones were analysed 3 weeks later; these showed only an 8.6% increase of integrated proviruses (arrows in Fig. 4c). These data would suggest that extrachromosomal HTLV-I DNA is capable of reintegrating into the host cell genome.

DISCUSSION

There are several possible mechanisms to explain the increase in copy number of integrated proviruses in the HTLV-I-infected HL60 cells. First, the virus may reinfect through virus
Fig. 3. Southern blot analyses of subclones obtained by further cloning of those clones studied in Fig. 2. Total cellular DNA digested by EcoRI was hybridized with a full-length HTLV-I probe. (a) Subclones of h-3 (one of the subclones of h; lane 1 of Fig. 2d). (b) Subclones of 35-2 (lane 3 of Fig. 2b). (c) Subclones of 35-3 (lane 4 of Fig. 2b). (d) Subclones of 35-15 (lane 14 of Fig. 2b. (e) Southern blot analysis of some of the subclones in (b) to (d) after 2 weeks of further culture. The numbers above the gel in (e) indicate the subclone number and the corresponding lanes in gels (b) to (d). The arrows in (d) indicate extrachromosomal proviruses. Large arrow indicates linear form, and small arrows indicate closed circular form of proviruses. Asterisks in (d) indicate subclones which were used for further recloning experiments in Fig. 4. Size markers as in Fig. 2.

particles released into the culture medium or through cell-to-cell contact; second, there may be reverse transcription of genomic viral RNA transcribed from the integrated provirus genome, followed by integration of the new provirus into the same host cell.

The first possibility is unlikely because of two observations. Uninfected HL60 cells cultured in the medium of HTLV-I-infected HL60 clone 35 (1:1 mixture with fresh medium) for 3 weeks did not show detectable extrachromosomal HTLV-I in the Hirt superantant from 1 × 10^7 cells (data not shown). Moreover, none of the 40 HL60 clones obtained from this culture by limiting dilution showed any integrated proviruses (data not shown). This finding correlates with the clinical observation that HTLV-I transmits by transfusion of blood cell components but not by serum components (Okochi et al., 1984). Virus production and release from HTLV-I-infected cells seem to be very low. Reinfection of the virus through cell-to-cell contact is also a remote
possibility although not formally excluded. However, transfer of HTLV-I from HTLV-I-infected IMR90 cells to uninfected HL60 cells by 3 months of coculture, selecting only tightly packed cell spheroids, resulted in a very low efficiency of successful transmission (less than 30% of clones were positive for integrated proviruses; see Fig. 1 of Hiramatsu et al., 1986). This efficiency of virus transmission is too low to explain the rather high rate of increase in copy number of integrated proviruses observed in our experiment (Fig. 2).

In support of the second possibility, the amount of extrachromosomal provirus was not influenced by culturing HTLV-I-infected HL60 cells at different cell densities (Fig. 5); on the basis of these observations, we favour the latter possibility. It is possible that the transient increase of virus production or the decrease of virus interference in the host cell provoked reinfection and integration of new proviruses.

A strong correlation between the increase of integrated proviruses and persistent extrachromosomal proviruses was observed. It should be noted, however, that the copy number of extrachromosomal provirus is usually maintained around 0·1 copy/cell. This indicates that
Fig. 5. Extrachromosomal proviruses in clone 35-2 cultured at different cell densities. Hirt supernatant DNA was prepared from 10⁷ cells after 5 days of culture at initial densities of 1 × 10⁶ cells/ml (lane 1) and 1 × 10⁵ cells/ml (lane 2). In the former, two-thirds of the medium was exchanged daily to maintain cell viability. Final cell density was 6 × 10⁶ cells/ml and 3 × 10⁵ cells/ml, respectively. The blot was hybridized with HTLV-I whole genomic probe. Large arrow indicates linear form and small arrow indicates closed circular form of extrachromosomal proviruses. Size markers as in Fig. 2.

less than 10% of the whole cell population harbours extrachromosomal proviruses. Even if we assume 100% efficiency of integration into the host cell genome for these molecules we cannot expect more than 10% of the progeny population to have newly acquired integrated proviruses. Results of subcloning experiments supported this reasoning. The lack of an increase in the provirus copy number among subclones of clone 1c (reported elsewhere) could also be explained by the fact that it did not harbour any detectable extrachromosomal HTLV-I DNA (Hiramatsu et al., 1986). Therefore, we consider that a spontaneous increase of extrachromosomal HTLV-I DNA must have preceded the recloning carried out in the experiment described in Fig. 2. In other words, the transient increase of extrachromosomal DNA (Fig. 3) was not the first event but was preceded by at least one similar event. This recurrent nature leads us to speculate that the phenomenon was regulated or determined by the epigenetic status of the host cell in culture. Moreover, the fact that the increase in extrachromosomal HTLV-I DNA, in spite of its rare occurrence, was observed simultaneously in three independent cultures of subclones derived from clone 35 (Fig. 3) suggests that the environment has an effect on the virus–host cell interaction. Certain changes in culture conditions might be responsible for the above increase of extrachromosomal HTLV-I DNA. Also, the phenomenon was observed in 30% to 50% of sub-subclones of two subclones (Fig. 3). This frequency is too high to be due to the appearance of mutations which are permissive for proviral DNA increase. The concept that the epigenetic condition of the host cell changes during culture is supported by the observation that HL60 cells, which are known to be bipotential precursors of monocytes and granulocytes, tend to differentiate spontaneously during culture so that around 10% of the population is phenotypically distinct from the rest (Momoi et al., 1986).
The mechanism for the abrupt increase in extrachromosomal HTLV-I DNA (Fig. 2) is still not clear. There were no obvious changes in the culture conditions employed in the experiment that could cause this phenomenon, although the cultured cells may be responsive to very subtle changes. An attempt to determine the conditions which provoke amplification of extrachromosomal HTLV-I DNA is now being undertaken. The increase of integrated proviruses during culture of HTLV-I-infected cells due to reintegration of the persistent extrachromosomal proviruses is not confined to our experimental system. Adult T cell leukaemia (ATL) cells harbour only a limited number of integrated proviruses (Yoshida et al., 1984), whereas ATL cells adapted to culture in vitro contain multiple copies (Hoshino et al., 1983). We think this increase occurred during the process of adaptation of ATL cells to culture conditions in vitro. During the adaptation period, activation of the transcription and expression of the latent HTLV-I proviruses has been reported (Hoshino et al., 1983). It would not be surprising if there was a concomitant appearance of extrachromosomal DNA during this period, and that this then reintegrated into the genome of the established ATL cell lines. Given these observations, the phenomenon of provirus amplification via reintegration of persistent extrachromosomal proviruses could be a general occurrence. We think this is one of the natural strategies used in the retrovirus life cycle to increase mutagenesis and induce alteration of host cell function.

We thank Mr J. Kaku who is a dental student of Tokyo Medical and Dental University for the statistical analysis of Poisson distribution. This work was supported by a grant-in-aid from the Ministry of Education.

REFERENCES


(Received 3 August 1987)