Molecular Cloning of Two Paralytogenic, Temperature-Sensitive Mutants, ts1 and ts7, and the Parental Wild-Type Moloney Murine Leukemia Virus

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Both spontaneous and bromodeoxyuridine-induced temperature-sensitive (ts) mutants have been isolated from Moloney leukemia virus (MoMuLV). A group of these ts mutants, ts1, ts7, and ts11, each of which was isolated on a separate occasion, was shown to be capable of inducing a rapidly fatal lower motor-neuron disease in inoculated mice. The pathogenicity induced by the group of mutants therefore differs strikingly from that of the other ts mutants. The mutant virus, tsl-19 and ts7, the paralytogenic, temperature-sensitive variant of MoMuLV-TB, together with their wild-type parent, MoMuLV-TB, were molecularly cloned. ts1-19, ts7-22, and wt-25, the infectious clones obtained on transfection to NIH/3T3 cells of the lambda Charon 21A recombinants of ts1, ts7, and wt, were found to have retained the characteristics of their non-molecularly cloned parents. In contrast to the wt virus, ts1-19 and ts7-22 are temperature-sensitive, inefficient in the intracellular processing of Pr80°wt at the restrictive temperature, and able to induce paralysis in CFW/D mice. Like the non-molecularly cloned ts7, the ts7-22 virus was also shown to be heat labile. The heat lability of the ts7 virion distinguishes it from ts1. Endonuclease restriction mapping with 11 endonucleases demonstrated that the base composition of MoMuLV-TB differs from that of the standard MoMuLV, but no difference was detected between the molecularly cloned ts1 and ts7 genomes. However, ts1 and ts7 differ from MoMuLV in the loss or acquisition of four different restriction sites, whereas they differ from MoMuLV-TB in the loss or acquisition of three different restriction sites.

As a first step in identifying the change(s) in ts1 and ts7 genomes which confer on them temperature sensitivity, inefficiency in the processing of Pr80°wt, and the ability to induce paralysis, ts1, ts7, and MoMuLV-TB were molecularly cloned into lambda Charon 21A, and the clones were characterized.

MATERIALS AND METHODS

Cells. Viruses were propagated in mouse TB cells, an established tissue culture cell line derived from mixed culture of thymus and bone marrow from CFW/D mice (1), and assayed on 15F cells (19). NIH/3T3 cells were used for transfection experiments. All cell lines were maintained in Dulbecco modified Eagle minimum supplemented with 8% fetal calf serum.

Viruses and virus assay. The strain used in this study (MoMuLV-TB) was isolated as described by Wong et al. (16). Since its isolation, MoMuLV-TB has been propagated in TB cells. It has also been single-virus-single-cell cloned on several occasions. Clone LV30, used in the present studies, is one of the isolates obtained in the most recent clonal isolation.

Shields et al. (10) have shown and we have confirmed that MoMuLV-TB can be distinguished from the standard MoMuLV by the electrophoretic mobility of the p30 protein. In this report, the two strains of MoMuLV are referred to as MoMuLV-TB and MoMuLV.

ts1 and ts7 are both spontaneous ts mutants of MoMuLV-TB that were isolated as described by Wong et al. (15, 16). They were isolated on separate occasions. The ts1 used in the present studies was also recently cloned and purified. ts7, however, was not recently single-virus-single-cell cloned before its cloning into lambda Charon 21A. The 15F assay for MuLV has been described previously (18).

Isolation and molecular cloning of unintegrated circular proviruses of ts1, ts7, and MoMuLV-TB. Circular proviruses of ts1, ts7, and MoMuLV were isolated (4) from TB cells repeatedly infected with virus at a titer of at least 10⁶ IU/ml.
Closed-circular proviruses were purified by centrifugation in CsCl-ethidium bromide gradients. The DNA in the fraction below the linear DNA band and above the pelleted RNA of each gradient was cloned into Charon 21A arms at the HindIII site essentially as reported by Berns et al. (2).

Molecularly cloned MoMuLV DNA, p8.2, an infectious permuted MoMuLV genome cloned into pBR322 at the HindIII site (9), was a gift from D. Baltimore. On receipt, the p8.2 DNA was transformed into HB101 and the recombinant plasmid DNA was purified. The molecularly cloned viral DNA of p8.2 will be referred to as wt-8.2.

**Analysis of viral DNA.** Small-scale purification of phage recombinant DNAs was carried out by the procedure of Shoemaker et al. (12). Restriction enzymes were purchased from Bethesda Research Laboratories, and the conditions for endonuclease digestions were as recommended by the supplier. Gel transfer and filter hybridization were carried out by the method of Southern (13).

**DNA transfection.** The calcium phosphate precipitation method of Graham and van der Eb (3) was used. At 6 to 18 h after the addition of the calcium-precipitated DNA, the cell monolayers were lightly trypsinized, rinsed with medium, and allowed to grow in complete medium until confluent. The cells were then passed into culture flasks. The supernatant was first assayed on 15F cell monolayers 7 to 9 days after transfection. The transfected cells were passed as needed. The transfected DNA was considered noninfectious if infectivity was not detected by the 15F assay about 1 month after transfection.

**Metabolic labeling of cells, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of viral proteins.** Immunoprecipitation of intracellular virus-specific proteins and sodium dodecyl sulfate-polyacrylamide gel electrophoresis were performed as described previously (17, 18). Gels were fluorographed and exposed to X-ray film at -70°C.

**Mouse strain.** The inbred CFWD mice used in this study were bred from a stock kindly provided by J. K. Ball and J. A. McCarter, University of Western Ontario, London, Ontario, Canada. The mice were maintained under strict thermal and photoperiod regulation. Feed and water were provided ad libitum in cages allowing both paralytic and nonparalytic mice to have free access to the feed and water.

**Inoculation procedure.** Neonate CFWD mice were injected within 48 h of birth with 100 µl of the virus stock (10⁶ to 10⁷ IU/ml). Virus was introduced into the peritoneal cavity with a tuberculin syringe fitted with a 25-gauge needle.

**RESULTS**

**Molecular cloning of ts1, ts7, and MoMuLV-TB.** Circular proviruses of ts1, ts7, and MoMuLV-TB were cloned into Charon 21A arms at the HindIII site. Thirty-four ts1 recombinants with full-size viral inserts were isolated. Seven recombinant clones, ts8-1-2, -19, -20, -22, -28, -30, and -31, were plaque purified for further characterization.

Of the 30 recombinants of MoMuLV-TB obtained, 6 selected at random were further studied. Four recombinants, wt-4, -14, -17, and -25, which contained full-size viral inserts, were selected for further characterization.

In the molecular cloning of ts7, 9 of the 29 recombinants, ts7-3, -4, -5, -6, -7, -8, -9, -22, and -24, containing full-size viral inserts, were further characterized.

**Infectivity of the molecularly cloned ts1, ts7, and wt MoMuLV-TB DNAs.** The recombinant DNAs ts8-1-2, -19, -20, -22, -28, -30, -31, ts7-2-2, -3, -5, -6, -7, -8, -9, -22, -24, and wt-4, -14, -17, and -25 were cleaved with HindIII, and the viral inserts were purified, religated, and transfected as described above. ts1-19, -20, -22, -28, -30, -31, ts7-3, -6, -7, -22, wt-17, and -25 DNAs produced infectious virus on transfection. No infectious virus was obtained for the rest of the DNAs transfected.

**Temperature sensitivity.** Infectious virus obtained from the ts1-19, ts7-22, and wt-25 transfected cultures were assayed in 15F cells at both the permissive (34°C) and the restrictive (39°C) temperatures. Since the foci induced in 15F cells depend on viral spread, this assay measures the replication efficiency of the virus examined. ts1-19 and ts7-22 produced approximately 3,000-fold and 43,000-fold more foci at 34 than at 39°C, respectively, whereas the difference between the number of foci produced by wt-25 at 34°C and 39°C was only 1.4-fold (Table 1).

**Heat lability.** It has been shown previously (15) that, in contrast to the parental wt virus, the virions of ts7 are heat labile at 39°C. The infectious virus produced by the ts7-22 transfected cultures were therefore tested for their heat lability. Virions were incubated at 39°C for 3 h. The amount of infectivity remaining at hourly intervals was determined by assaying in 15F cells at the permissive temperature. Virions from ts1-19 and wt-25 DNA transfected cultures were similarly tested. The virions produced by wt-25 and ts1-19 transfected cells were much less heat labile than those produced by the ts7-22 DNA transfected culture (Fig. 1). The infectivity of wt-25 and ts1-19 decreased only about fivefold in 3 h on incubation at 39°C, whereas the infectivity of ts7-22 decreased by more than 2,000-fold over the 3-h period.

**Processing of Pr80°** in infected cells. We have previously demonstrated (17) that the paralytogenic ts mutants ts1, ts7, and ts11 are inefficient in the processing of Pr80° at the restrictive temperature. The infectious virus-producing NIH/3T3 cell cultures obtained by transfecting molecularly cloned ts1-19, ts7-22, and wt-25 DNAs were therefore processed to determine the efficiency of the intracellular processing of Pr80°.

Although the processing of Pr65° appears normal when ts1-19 transfected cells grown at the restrictive temperature is greatly retarded when compared with the processing of Pr80° in wt-25 transfected cells during the 3-h chase period (Fig. 2). The pattern of processing of Pr80° in ts7-22 transfected cells is similar to that of ts1-19, and the data are therefore not shown.

**Ability to induce paralysis in CFWD mice.** A common characteristic of ts1 and ts7 is their ability to induce paralysis when injected into neonate CFWD mice. The infectious viruses produced by the NIH/3T3 cells transfected with the molecularly cloned DNAs of ts1-19, -20, -28, -30, -31, ts7-22, and wt-25 were assayed in NIH/3T3 cells at the permissive (34°C) and the restrictive (39°C) temperatures. The 15F assay for virus infectivity was done as described previously (18).

**TABLE 1. Comparison of virus titers when assayed at the permissive (34°C) and the nonpermissive (39°C) temperatures**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Titera (infectious units/ml) at:</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34°C</td>
<td>39°C</td>
</tr>
<tr>
<td>wt-25</td>
<td>1.4 x 10⁶</td>
<td>1.0 x 10⁶</td>
</tr>
<tr>
<td>ts1-19</td>
<td>5.4 x 10⁷</td>
<td>1.8 x 10⁷</td>
</tr>
<tr>
<td>ts7-22</td>
<td>5.2 x 10⁷</td>
<td>1.2 x 10⁸</td>
</tr>
</tbody>
</table>

a The supernatant of the infectious virus producing ts1-19, ts7-22, or wt-25 DNA transfected cultures grown at 34°C was adsorbed onto 15F cells at 34°C for 45 min, then incubated at the permissive (34°C) and restrictive (39°C) temperatures. The 15F assay for virus infectivity was done as described previously (18).
and wt-25 as well as with the non-molecularly cloned ts1, ts7, and MoMuLV-TB viruses were injected into CFW/D mice within 48 h after birth. The results of these studies are shown in Table 2. Every mouse injected with the non-molecularly cloned ts1 virus and the molecularly cloned ts1-19 virus ranged from 20 to 90 days post-injection, about 78 and 63%, respectively, of the mice injected with these viruses became paralyzed by 40 days post-injection (Fig. 3). By 60 days post-injection, over 80% of the mice had become paralyzed. Although the latent period for both the non-molecularly cloned ts1 virus and the molecularly cloned ts1-19 virus ranged from 20 to 80 days post-injection, for both of these viruses there appears to be a more protracted latent period. Over 60% of the 30 mice injected with the non-molecularly cloned ts7 virus became paralyzed from 40 to 60 days post-injection. In the case of the molecularly cloned ts7-22 virus, for which only 16 mice were tested, although about 30% of the mice became paralyzed for 40 days post-injection, the majority became paralyzed from 50 to 80 days post-injection. In contrast, mice injected with the non-molecularly cloned MoMuLV-TB virus and the cloned wt-25 virus were not paralyzed (Table 2). These mice lived for at least 190 days after inoculation with either of these viruses. These studies clearly showed that the ability to induce paralysis is genetically determined and is due to mutation(s) in the ts1 and ts7 genomes.

Endonuclease restriction mapping. In an attempt to correlate the unique biological characteristics of ts1 and ts7 with alterations in their genomic composition, restriction maps of the infectious molecularly cloned viral DNAs from ts1-28, ts7-22, and wt-25 DNAs were constructed and compared with that of molecularly cloned standard MoMuLV DNA (wt-8.2) from p8.2.

Since another approach in identifying the genomic segment responsible for a particular phenotype of the mutant is that of constructing chimeric genomes between the mutant and wt DNAs, we have fragmented the viral genomes of ts1-19, wt-25, and wt-8.2 into two halves: (i) a Sma-HindIII segment from nucleotide 31-4894 (numbering of the bases is based on the method of Shinnick et al. [11]), which consists of the major portion of the pol gene, all of the gag gene, U5,

![Graph](image1.png)

FIG. 1. Heat lability of infectious virus. Supernatant harvested from ts1-19, ts7-22, and wt-25 transfected NIH/3T3 cell cultures grown at 34°C was incubated at 39°C. Samples were collected at the times indicated, and the titer of the virus in the supernatant surviving incubation at 39°C was determined by 15F assay as described previously (18).

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. of mice injected</th>
<th>% Paralyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts1</td>
<td>59</td>
<td>100</td>
</tr>
<tr>
<td>ts1-19</td>
<td>28</td>
<td>100</td>
</tr>
<tr>
<td>ts1-20</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>ts1-28</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>ts1-30</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>ts1-31</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>ts7</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>ts7-22</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>MoMuLV-TB</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>wt-25</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mice less than 48 h old were injected intraperitoneally with 10^7 to 10^8 infectious units of virus in 0.1 ml of growth medium.
BamHI, and a portion of the R region of the long terminal repeat (LTR); and (ii) a HindIII-PstI segment from nucleotides 4895–8264 and 1–567, which consists of the remaining portion of the pol gene, all of the env gene, the LTR, and the amino-terminal portion of the gag gene. The SmaI-HindIII segments were cloned into pKC7 (7), and the HindIII-PstI segments were cloned into pUC9 (14). Details concerning the construction of these recombinant plasmids are reported elsewhere. Chimeric genomes derived by ligating the SmaI-HindIII segment of ts1-19 to the HindIII-PstI segment of wt-25 or wt-8.2 and vice versa were found to be infectious (P. H. Yuen, D. Malehorn, C. Knupp, and P. K. Y. Wong, J. Virol., in press). To construct and compare the restriction maps of ts1-19, wt-25, and wt-8.2, the HindIII-PstI segment of each virus was isolated from its respective plasmid vectors. The SmaI-HindIII viral DNA segments were cleaved from their plasmid vectors with KpnI, which yielded for each virus a ca. 2.9-kilobase-pair (kb) KpnI-KpnI segment from nucleotide 37–2862 and a ca. 2.0-kbp KpnI-HindIII segment from nucleotide 2863–4894. It was necessary to restrict the SmaI-HindIII fragment with HindIII and KpnI instead of HindIII and SmaI because the viral SmaI-HindIII fragment was too similar in size to the vector SmaI-HindIII fragment. KpnI was judged to be the appropriate enzyme to use in the double digest, since it cleaves twice and one of the cleavages is within 6 bases of the SmaI site in the U5 region of the LTR. These DNA fragments were individually purified and used in the studies reported here. The HindIII-PstI, KpnI-KpnI, and KpnI-HindIII segments of each virus studied were restricted with the following enzymes: AvaI, BamHI, BglII, ClaI, HpaI, PvuII, SacI, SmaI, XbaI, and Xhol. The HindIII-PstI DNA sequences were also restricted with KpnI, whereas the KpnI-KpnI and HindIII-PstI segments were also restricted with PstI. Figures 4 and 5 show some of the restriction patterns obtained.

The restriction pattern of the HindIII-PstI fragment of MoMuLV is as predicted from the nucleotide sequence map of Shinnick et al. (11), except that ClaI did not cleave at nucleotide 4981 as predicted.

Endonuclease digests of the HindIII-PstI fragment (Fig. 4A through C) showed that the restriction patterns obtained only differ among ts1-19, wt-25, and wt-8.2 when the HindIII-PstI segments were restricted with XbaI. Figure 4C, lane 4, shows the four fragments of about 0.43, 0.44, 0.72, and 2.3 kbp predicted from the nucleotide sequence of MoMuLV (11). The 0.43- and 0.44-kbp fragments are shown migrating as a single unit in lane 4. Two faintly staining fragments of about 3 and 0.9 kbp are also present. These are probably the partial restriction products from the XbaI site at nucleotide 5767 to the PstI site at nucleotide 567 and the sequence from HindIII to the XbaI site at nucleotide 5766. Lane 5 shows the XbaI restriction pattern of wt-25 DNA. Although the 2.3- and 0.7-kbp fragments were present, the two 0.4-kbp fragments were replaced by a well-stained fragment the same size as the partially cleaved 0.9-kbp fragment shown in lane 4. The absence of the 0.43- and 0.44-kbp fragments and the presence of the 0.9-kbp fragment may be accounted for by the loss of the XbaI site at nucleotide 5325 of wt-25 DNA (represented as MoMuLV-TB in Fig. 6). Lane 6 shows the XbaI restriction pattern of the ts1-19 DNA. In this case, only the 0.9-kbp fragment present in the XbaI restriction of wt-25 DNA is retained. However, a fragment the same size as the faintly staining 3.0-kbp fragment shown in lane 4 is present. These observations suggest that the ts1-19 DNA may have lost not only the XbaI site at nucleotide 5325, but also the XbaI site at nucleotide 8113 (Fig. 6). To confirm this possibility, the HindIII-PstI DNA fragment of ts1-19 was digested with both XbaI and PvuII. As predicted from the nucleotide sequence of MoMuLV, and confirmed in the present study, PvuII restriction of the HindIII-PstI DNA fragment of MoMuLV, MoMuLV-TB, and ts1-19 generated four fragments of about 2.8, 0.8, 0.2, and 0.1 kbp. Only the two larger fragments are visible in Fig. 4C, lanes 1 through 3. If the XbaI site at nucleotide 8113 is missing in the ts1-19 DNA, then restriction of the ts1-19 DNA with both XbaI and PvuII should reduce the 3.0-kbp fragment of ts1-19 to a size similar to that of the largest fragment obtained for the XbaI and PvuII digests of wt-25 and wt-8.2 DNAs, since the PvuII cleavage sites are located downstream from the XbaI site at nucleotide 8113. The predicted results are clearly shown in Fig. 4C, lanes 7 through 9.

The above studies showed that the HindIII-PstI DNA segments of both the MoMuLV-TB and ts1 genomes differ from that of MoMuLV in the loss of the XbaI site at nucleotide 5325. In addition, the HindIII-PstI segment of the ts1 genome has lost the XbaI site present at nucleotide 8113 in both of the wt genomes.

The HindIII-PstI segment of the molecularly cloned infectious DNA of ts1-20, another Charon 21A ts1 recombinant obtained in a separate cloning experiment, and ts7-22 were isolated and digested with the same endonuclease restriction enzymes. The endonuclease restriction map of the HindIII-PstI genomic fragments of ts1-20 and ts7-22 cannot be distinguished from that of ts1-19 (data not shown).

Results of the endonuclease digests of the KpnI-KpnI genomic segments of MoMuLV, MoMuLV-TB, and ts1 from nucleotide 37–2862 and the KpnI-HindIII segments from nucleotide 2863–4894 are shown in Fig. 5A through D. Differences between the genomes were only observed in the BamHI and BglII digests of the KpnI-KpnI DNA segments and the SacI and Xhol digests of the KpnI-HindIII segments.
Figure 5A, lane 2 shows the expected 1.9- and 1.0-kbp bands on BgIII cleavage of the KpnI-KpnI fragment of MoMuLV at nucleotide 1906. In contrast, the homologous DNA segments of both ts1 and MoMuLV-TB were not cleaved by BgIII (lanes 4 and 6).

No BamHI site was found in the KpnI-KpnI DNA segments of MoMuLV (Fig. 5B, lane 2) and MoMuLV-TB (Fig. 5B, lane 4). In contrast, the homologous ts1 DNA was cleaved once, generating two fragments of approximately 2.2 and 0.6 kbp (Fig. 5B, lane 6). To locate the BamHI site in the ts1 DNA, the KpnI-KpnI ts1 DNA segment was digested with both BamHI and PstI. Figure 5D, lane 2, shows the expected ca. 2.1- and 0.5-kbp fragments on PstI digestion (the small 0.2-kbp PstI-PstI fragment is not visible on the ethidium bromide-stained gel). On cleavage with BamHI and PstI, the 0.5-kbp fragment remains present, but the ca. 2.3-kbp fragment is further cleaved, generating two fragments of approximately 1.5 and 0.6 kbp (lane 4), thus locating the BamHI site at about 0.6 kb from the 3' end of the KpnI-KpnI ts1 DNA segment.

Examination of the nucleotide sequence of MoMuLV at about 0.6 bp upstream from the KpnI site at nucleotide 2863 showed that, beginning at nucleotide 2355, there is a sequence GGACCC and, beginning at nucleotide 2380, there is another sequence GGTC (11). A single-base substitution in either sequence will generate a BamHI site. In both cases, although the amino acid residue will be changed there is no disruption of the reading frame. We have therefore tentatively assigned the BamHI site to either of these two sites.

On XhoI digestion, the KpnI-HindIII segment of MoMuLV-TB is cleaved (Fig. 5B, lane 11), which is in contrast to the absence of an XhoI site in both MoMuLV (Fig. 5B, lane 9) and ts1 (Fig. 5B, lane 13). To locate the XhoI site in the MoMuLV-TB DNA, the KpnI-HindIII DNA segment was doubly digested with XhoI and SalI. Restriction with SalI generated the expected two fragments of approximately 1.2 and 0.8 kbp (Fig. 5D, lane 6), since the SalI site is located at about 0.8 kbp from the KpnI site. On restriction with XhoI and SalI simultaneously, the smaller (0.8-kbp) fragment was further cleaved to yield two very similar-sized fragments of about 0.4 kbp (Fig. 5D, lane 7), indicating that the XhoI site is located about 0.4 kbp from the KpnI site. The other λ recombinant DNAs of MoMuLV-TB λwt-14 and λwt-17 were also restricted with XhoI, and both were found to possess the additional XhoI site in the KpnI-HindIII segment of the pol gene. Examination of the nucleotide sequence of MoMuLV (11) at about 0.4 kbp from

FIG. 4. Endonuclease digests of the HindIII-PstI restriction fragments from nucleotide 4894-8264 and 1-567 restriction fragments of wt-8.2 (panels A and B: lanes 1, 4, 7, and 10; panel C: lanes 1, 4, and 7); wt-25 (panels A and B: lanes 2, 5, 8, and 11; panel C: lanes 2, 5, and 8); ts1-19 (panels A and B: lanes 3, 6, 9, and 12; panel C: lanes 3, 6, and 9). M. HindIII-restricted fragments of λ and HaeIII digests of φX174. The endonuclease used were Smal (Sm), PstII (P), HpaI (Hp), AvaI (A), BamHI (B), BgIII (Bg), KpnI (K), and XhoI (X). Un. Unrestricted fragment.
FIG. 5. Endonuclease digests of the KpnI-KpnI sequence from nucleotide 36-2862 of wt-8.2, wt-25, and tsl-19 (panels A, B, and C; even-numbered lanes) and the KpnI-HindIII sequence from nucleotide 2863-4894 of wt-8.2, wt-25, and tsl-19 (panels A, B, and C; odd-numbered lanes). M, HindIII-restricted fragments of λ and HaeIII digests of φX174. The endonucleases used were BglII (Bg), PvuII (P), PstI (Ps), BamHI (B), XhoI (Xh), SmaI (Sm), and Sall (S).

wt-8.2 DNA: panel A, lanes 2, 3, 8, 9, 14, and 15; panel B, lanes 2, 3, 8, 9, 15, and 16; and panel C, lanes 4, 5, 10, and 11. wt-25 DNA: panel A, lanes 4, 5, 10, 11, 16, and 17; panel B, lanes 4, 5, 10, 11, 17, and 18; panel C, lanes 4, 5, 10, and 11. tsl-19 DNA: panel A, lanes 6, 7, 12, 13, and 19; panel B, lanes 6, 7, 12, 13, 19, and 20; panel C, lanes 6, 7, 12, and 13. Panel D, lanes 2 through 4: KpnI-KpnI DNA sequence of tsl-19 restricted with BamHI (lane 2), PstI (lane 3), and BamHI and PstI (lane 4); lanes 5 through 10: KpnI-HindIII DNA sequence of wt-25 restricted with XhoI (lane 5), Sall (lane 6), XhoI and Sall (lane 7), Sall (lane 8), Sall (lane 9), and Sall and Sall (lane 10). M, HindIII restriction fragments of λ and HaeIII restriction fragments of φX174.

The KpnI site showed that, beginning at nucleotide 3316 and 3345, there is a sequence CTCGGG which is an AvaI recognition site. Substituting the G residue at nucleotide 3320 or 3349 with an A residue will generate an XhoI recognition site while retaining the AvaI recognition site. We have therefore tentatively assigned the XhoI site to one of the AvaI sites (Fig. 6). In the first case, no change in amino acid sequence will result; in the second case, a codon for glycine becomes a codon for glutamic acid.

The KpnI-KpnI and KpnI-HindIII segments of the molecularly cloned DNA of tsl-20 and ts7-22 were isolated and similarly endonuclease restriction mapped. The infectious
DNA of tsl-20 and ts7-22 could not be distinguished from that of tsl-19 at this level of endonuclease restriction analysis.

The restriction maps of the molecularly cloned DNAs of wt-8.2, wt-25, tsl-19, and ts7-22 of standard MoMuLV, MoMuLV-TB, tsl-1, and ts7, respectively, are schematically presented in Fig. 6.

**DISCUSSION**

To identify the change(s) in the genome which confers on tsl and ts7 the temperature sensitivity, inefficiency in the processing of Pr80\textsuperscript{env}, and the ability to induce hind-limb paralysis, we have molecularly cloned tsl-1, tsl-7, and MoMuLV-TB. The infectious viruses obtained on transfection of the molecularly cloned tsl-19, ts7-22, and wt-25 DNAs into NIH/3T3 cells were found to have retained the characteristics of their non-molecularly cloned parents.

Endonuclease restriction mapping with 11 endonucleases demonstrated that the base composition of MoMuLV-TB differs from that of MoMuLV (Fig. 6). It has been reported by Shields et al. (10) and confirmed by us (unpublished data) that the electrophoretic mobility of MoMuLV-TB p30 differs from that of MoMuLV. We have also found that the p30 of tsl and ts7 has the same electrophoretic mobility as the p30 of MoMuLV-TB (17). The BglII site present at nucleotide 1905 at the 5' end of the p30 coding sequence of MoMuLV is absent in the MoMuLV-TB, tsl-1, and tsl-7 genomes. This finding clearly shows that this segment of the genome is mutated. However, whether the mutation at the BglII site in the MoMuLV-TB, tsl-1, and ts7 genomes is responsible for the difference in electrophoretic mobility of p30 remains to be investigated.

Another characteristic that distinguishes standard MoMuLV from MoMuLV-TB is the relatively longer latent period before mice infected with MoMuLV-TB die from lymphoma (5) compared with mice infected with MoMuLV (6). We have shown that CFW/D mice inoculated with either the non-molecularly cloned MoMuLV-TB or the cloned wt-25 virus live for at least 190 days. In another experiment (Yuen et al., in press), CFW/D mice injected with the infectious virus obtained from the construct consisting of the 3' pol-env-LTR fragment of MoMuLV and the 5' U5-gag-pol of tsl succumbed to lymphoma 2.5 to 4 months post-injection. These findings indicate that the course of leukemogenicity is controlled by the pol-env-LTR genomic sequence. Comparison of the restriction map of the 3' pol-env-LTR sequences of MoMuLV with those of MoMuLV-TB showed that MoMuLV-TB differs from MoMuLV in the loss of the SacI site in U3 and the loss of the Xbal site in the 3' end of the pol coding sequence. Whether the effects of these and other presently undetermined differences on the potency of the virus in the induction of lymphoma are significant remains to be investigated. The differences further emphasize that MoMuLV-TB has diverged from MoMuLV.

Endonuclease restriction maps of tsl-1 and ts7 obtained with 11 enzymes were similar. This is not altogether unexpected. Despite the fact that tsl-1 and ts7 were isolated on separate occasions, they share certain common characteristics. However, they are distinguishable in that mice infected with ts7 need a slightly longer latent period before developing paralysis than those infected with tsl (Fig. 3) and that the virions of ts7 are more heat labile than those of tsl (Fig. 1). A more detailed analysis of these genomes is now in progress to identify the base change(s) which confers on ts7 virions heat lability and the attenuated ability to induce paralysis.

ts1 and ts7 differ from MoMuLV in the loss or acquisition of four different restriction sites, whereas tsl and ts7 differ from MoMuLV-TB in the loss or acquisition of three different restriction sites. However, only two changes are peculiar to ts1 and ts7 and not shared with either of the wt genomes. These are the loss of the Xbal site at nucleotide 8113 and the presence of an additional BamHI site at the 5' end of the pol coding sequence (Fig. 6). This finding is unexpected, since studies reported elsewhere (Yuen et al., in press) showed that the HindIII-BamHI genomic sequence of ts1 from nucleotide 4894 to 6538 was found to determine the temperature sensitivity, processing of Pr80\textsuperscript{env}, and possibly the ability to induce paralysis. Studies are now in...
progress to determine by exchanging genomic fragments within the \textit{HindIII-BamHI} sequence and nucleotide sequencing whether the \textit{ts} function, the inefficiency in the processing of \textit{Pr80}^{env}, and the ability to induce paralysis result from a single mutation or multiple mutations.

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**LITERATURE CITED**


