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Molecular Cloning of Two Paralytogenic, Temperature-Sensitive Mutants, *ts*1 and *ts*7, and the Parental Wild-Type Moloney Murine Leukemia Virus

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ts1 and ts7, the paralytogenic, temperature-sensitive mutants of Moloney murine leukemia virus (MoMuLV), together with their wild-type parent, MoMuLV-TB, were molecularly cloned. ts1-19, ts7-22, and wt-25, the infectious viruses 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 $Pr80^{env}$ at the restrictive temperature, and able to induce paralysis in CFW/D mice. Like the non-molecularly cloned ts7, the ts7-22 virion 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.

Both spontaneous and bromodeoxyuridine-induced temperature-sensitive (*ts*) mutants have been isolated from MoMuLV-TB, a strain of Moloney murine leukemia virus (MoMuLV) (8, 15, 16). A group of these *ts* mutants, *ts*1, *ts*7, and *ts*11, each of which was isolated on a separate occasion, was shown to be capable of inducing a rapidly fatal lower motor-neuron disease on inoculation into neonates of susceptible strains of mice such as CFW/D and BALB/c (5, 17). The pathogenicity induced by this group of mutants therefore differs strikingly from that of the T-cell lymphomas induced by the parental wild-type (wt) MoMuLV-TB. Furthermore, another *ts* mutant of MoMuLV-TB, *ts*3, which is characterized by a different phenotype from that of *ts*1, *ts*7, and *ts*11, failed to induce hind-limb paralysis in these susceptible strains of mice. These findings suggest that the ability of *ts*1, *ts*7, and *ts*11 to induce hind-limb paralysis may be due to a specific alteration of the viral genome and is not a common characteristic of all *ts* mutants of MoMuLV.

Biochemical studies (17) showed that at the restrictive temperature (39°C) these paralytogenic mutants are all defective in the processing of the envelope precursor protein $Pr80^{env}$, so that this protein accumulates in the infected cells. Furthermore, the inefficient processing of $Pr80^{env}$ in the mutant-infected cells at the restrictive temperature can be reversed by shifting the culture to the permissive temperature (34°C). In contrast, the nonparalytogenic mutant, *ts*3, like the wt virus, shows normal processing of $Pr80^{env}$ in infected cells at 39°C.

Whether inefficiency in the processing of $Pr80^{env}$ and the ability to induce paralysis results from the same mutation or two different and unrelated mutations has not been determined. In addition, whether the accumulation of $Pr80^{env}$ in the infected cells plays a role in the induction of paralysis remains to be investigated.

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As a first step in identifying the change(s) in the *ts*1 and *ts*7 genomes which confer on them temperature sensitivity, inefficiency in the processing of $Pr80^{env}$, and the ability to induce paralysis, *ts*1, *ts*7, and MoMuLV-TB were molecularly cloned into lambda Charon 21A, and the cloned genomes 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.

*ts*1 and *ts*7 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 *ts*1 used in the present studies was also recently clonally purified. *ts*7, 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 *ts*1, *ts*7, and MoMuLV were isolated (4) from TB cells repeatedly infected with virus at a titer of at least 10^6 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 *Hin*dIII 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 *Hin*dIII 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 CFW/D 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 CFW/D mice were injected within 48 h of birth with 100 μl of the virus stock (10^6 to 10^7 IU/ml). Virus was introduced into the peritoneal cavity with a tuberculin syringe fitted with a 25-gauge needle.

RESULTS

Molecular cloning of *ts*1, *ts*7, and MoMuLV-TB. Circular proviruses of *ts*1, *ts*7, and MoMuLV-TB were cloned into Charon 21A arms at the *Hin*dIII site. Thirty-four *ts*1 recombinants with full-size viral inserts were isolated. Seven recombinant clones, λ*ts*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, λwt-4, -14, -17, and -25, which contained full-size viral inserts, were selected for further characterization.

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

Infectivity of the molecularly cloned *ts*1, *ts*7, and wt MoMuLV-TB DNAs. The recombinant DNAs λ*ts*1-2, -19, -20, -22, -28, -30, -31, λ*ts*7-2, -3, -5, -6, -7, -8, -9, -22, -24, and λwt-4, -14, -17, and -25 were cleaved with *Hin*dIII, and the viral inserts were purified, religated, and transfected as described above. *ts*1-19, -20, -22, -28, -30, -31, *ts*7-3, -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 *ts*1-19, *ts*7-22, 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. *ts*1-19 and *ts*7-22 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 *ts*7 are heat labile at 39°C. The infectious virus produced by the *ts*7-22 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 *ts*1-19 and wt-25 DNA transfected cultures were similarly tested. The virions produced by wt-25 and *ts*1-19 transfected cells were much less heat labile than those produced by the *ts*7-22 DNA transfected culture (Fig. 1). The infectivity of wt-25 and *ts*1-19 decreased only about fivefold in 3 h on incubation at 39°C, whereas the infectivity of *ts*7-22 decreased by more than 2,000-fold over the 3-h period.

Processing of $Pr80^{env}$ in infected cells. We have previously demonstrated (17) that the paralytogenic *ts* mutants *ts*1, *ts*7, and *ts*11 are inefficient in the processing of $Pr80^{env}$ at the restrictive temperature. The infectious virus-producing NIH/3T3 cell cultures obtained by transfecting molecularly cloned *ts*1-19, *ts*7-22, and wt-25 DNAs were therefore processed to determine the efficiency of the intracellular processing of $Pr80^{env}$.

Although the processing of $Pr65^{gag}$ appears normal, the processing of $Pr80^{env}$ in *ts*1-19 transfected cells grown at the restrictive temperature is greatly retarded when compared with the processing of $Pr80^{env}$ in wt-25 transfected cells during the 3-h chase period (Fig. 2). The pattern of processing of $Pr80^{env}$ in *ts*7-22 transfected cells is similar to that of *ts*1-19, and the data are therefore not shown.

Ability to induce paralysis in CFW/D mice. A common characteristic of *ts*1 and *ts*7 is their ability to induce paralysis when injected into neonate CFW/D mice. The infectious viruses produced by the NIH/3T3 cells transfected with the molecularly cloned DNAs of *ts*1-19, -20, -28, -30, -31, *ts*7-22,

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

Mutant	Titer[a] (infectious units/ml) at:		Ratio
	34°C	39°C	
wt-25	1.4×10^8	1.0×10^8	1.4
*ts*1-19	5.4×10^7	1.8×10^4	3.0×10^3
*ts*7-22	5.2×10^7	1.2×10^2	4.3×10^5

[a] The supernatant of the infectious virus producing *ts*1-19, *ts*7-22, 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 *ts*1, *ts*7, 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 *ts*1 and *ts*7 DNAs became paralyzed. Although the latent period for both the non-molecularly cloned *ts*1 virus and the molecularly cloned *ts*1-19 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 *ts*7 virus and the molecularly cloned *ts*7-22 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 *ts*7 virus became paralyzed from 40 to 60 days post-injection. In the case of the molecularly cloned *ts*7-22 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 *ts*1 and *ts*7 genomes.

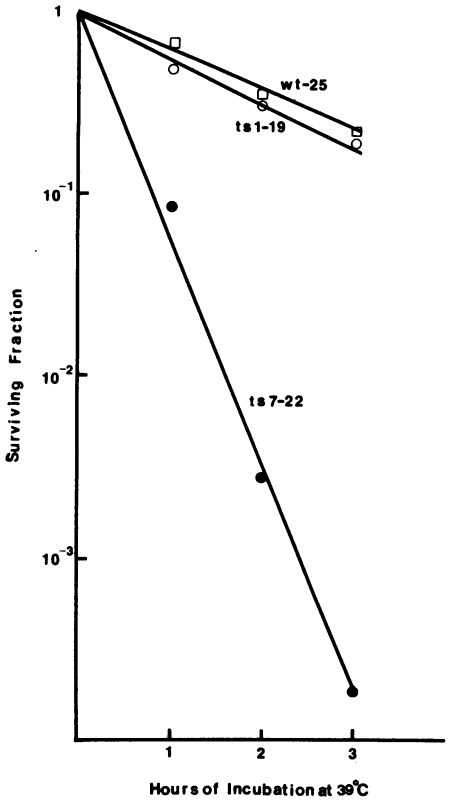

FIG. 1. Heat lability of infectious virus. Supernatant harvested from *ts*1-19, *ts*7-22, and wt-25 transfected NIH/3T3 cell cultures grown at 34°C was incubated at 39°C. Samples were collected at the times indicated, and the titer of the virus in the supernatant surviving incubation at 39°C was determined by 15F assay as described previously (18).

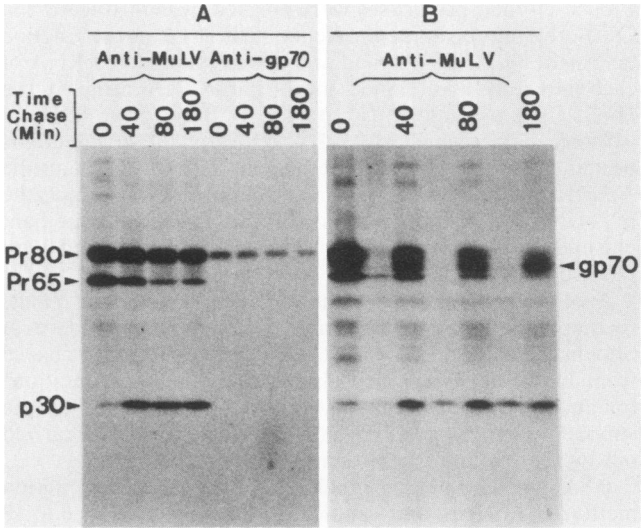

FIG. 2. Intracellular processing of $Pr80^{env}$ to *gp*70 in *ts*1-19 or wt-25 DNA transfected NIH/3T3 cells at the restrictive temperature (39°C). Infected cells were pulsed for 15 min with [^{35}S]methionine, followed by 0-, 40-, 80-, and 180-min chase periods. Cell extracts were immunoprecipitated with antiserum to MoMuLV, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, fluorographed, and exposed to Kodak X-ray film. Panel A, *ts*1-19 transfected cells grown at 39°C. Panel B, wt-25 transfected cells grown at 39°C.

Endonuclease restriction mapping. In an attempt to correlate the unique biological characteristics of *ts*1 and *ts*7 with alterations in their genomic composition, restriction maps of the infectious molecularly cloned viral DNAs from λ*ts*1-19, λ*ts*7-22, and λwt-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 *ts*1-19, wt-25, and wt-8.2 into two halves: (i) a *Sma-Hin*dIII 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,

TABLE 2. Pathogenicity of CFW/D mice infected with virus produced by non-molecularly and molecularly cloned *ts*1, *ts*7, and wt viruses[a]

Virus	No. of mice injected	% Paralyzed
*ts*1	59	100
*ts*1-19	28	100
*ts*1-20	9	100
*ts*1-28	1	100
*ts*1-30	4	100
*ts*1-31	10	100
*ts*7	30	100
*ts*7-22	16	100
MoMuLV-TB	50	0
wt-25	16	0

[a] Mice less than 48 h old were injected intraperitoneally with 10^5 to 10^6 infectious units of virus in 0.1 ml of growth medium.

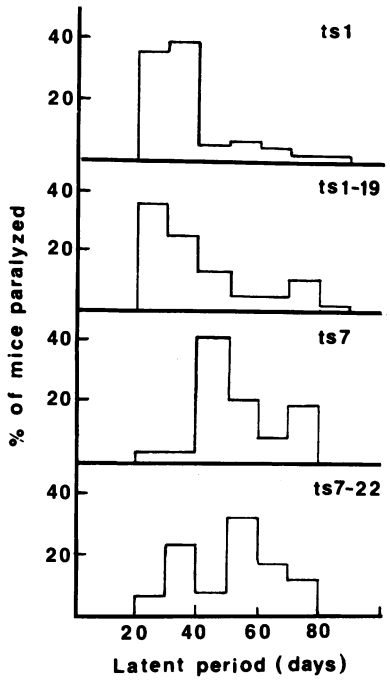

FIG. 3. Histogram of the percentage of mice paralyzed, with the indicated latent periods.

and a portion of the R region of the long terminal repeat (LTR); and (ii) a *Hin*dIII-*Pst*I 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 *Sma*I-*Hin*dIII segments were cloned into pKC7 (7), and the *Hin*dIII-*Pst*I segments were cloned into pUC9 (14). Details concerning the construction of these recombinant plasmids are reported elsewhere. Chimeric genomes derived by ligating the *Sma*I-*Hin*dIII segment of *ts*1-19 to the *Hin*dIII-*Pst*I 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 *ts*1-19, wt-25, and wt-8.2, the *Hin*dIII-*Pst*I segment of each virus was isolated from its respective plasmid vectors. The *Sma*I-*Hin*dIII viral DNA segments were cleaved from their plasmid vectors with *Kpn*I, which yielded for each virus a ca. 2.9-kilobase-pair (kbp) *Kpn*I-*Kpn*I segment from nucleotide 37–2862 and a ca. 2.0-kbp *Kpn*I-*Hin*dIII segment from nucleotide 2863–4894. It was necessary to restrict the *Sma*I-*Hin*dIII fragment with *Hin*dIII and *Kpn*I instead of *Hin*dIII and *Sma*I because the viral *Sma*I-*Hin*dIII fragment was too similar in size to the vector *Sma*I-*Hin*dIII fragment. *Kpn*I 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 *Sma*I site in the U5 region of the LTR. These DNA fragments were individually purified and used in the studies reported here. The *Hin*dIII-*Pst*I, *Kpn*I-*Kpn*I, and *Kpn*I-*Hin*dIII segments of each virus studied were restricted with the following enzymes: *Ava*I, *Bam*HI, *Bgl*II, *Cla*I, *Hpa*I, *Pvu*II, *Sac*I, *Sma*I, *Xba*I, and *Xho*I. The *Hin*dIII-*Pst*I DNA sequences were also restricted with *Kpn*I, whereas the *Kpn*I-*Kpn*I and *Hin*dIII-*Kpn*I segments were also restricted with *Pst*I. Figures 4 and 5 show some of the restriction patterns obtained.

The restriction pattern of the *Hin*dIII-*Pst*I fragment of MoMuLV is as predicted from the nucleotide sequence map of Shinnick et al. (11), except that *Cla*I did not cleave at nucleotide 4981 as predicted.

Endonuclease digests of the *Hin*dIII-*Pst*I fragment (Fig. 4A through C) showed that the restriction patterns obtained only differ among *ts*1-19, wt-25, and wt-8.2 when the *Hin*dIII-*Pst*I segments were restricted with *Xba*I. 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 *Xba*I site at nucleotide 5767 to the *Pst*I site at nucleotide 567 and the sequence from *Hin*dIII to the *Xba*I site at nucleotide 5766. Lane 5 shows the *Xba*I 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 *Xba*I site at nucleotide 5325 of wt-25 DNA (represented as MoMuLV-TB in Fig. 6). Lane 6 shows the *Xba*I restriction pattern of the *ts*1-19 DNA. In this case, only the 0.9-kbp fragment present in the *Xba*I 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 *ts*1-19 DNA may have lost not only the *Xba*I site at nucleotide 5325, but also the *Xba*I site at nucleotide 8113 (Fig. 6). To confirm this possibility, the *Hin*dIII-*Pst*I DNA fragment of *ts*1-19 was digested with both *Xba*I and *Pvu*II. As predicted from the nucleotide sequence of MoMuLV and confirmed in the present study, *Pvu*II restriction of the *Hin*dIII-*Pst*I DNA fragment of MoMuLV, MoMuLV-TB, and *ts*1-19 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 *Xba*I site at nucleotide 8113 is missing in the *ts*1-19 DNA, then restriction of the *ts*1-19 DNA with both *Xba*I and *Pvu*II should reduce the 3.0-kbp fragment of *ts*1-19 to a size similar to that of the largest fragment obtained for the *Xba*I and *Pvu*II digests of wt-25 and wt-8.2 DNAs, since the *Pvu*II cleavage sites are located downstream from the *Xba*I site at nucleotide 8113. The predicted results are clearly shown in Fig. 4C, lanes 7 through 9.

The above studies showed that the *Hin*dIII-*Pst*I DNA segments of both the MoMuLV-TB and *ts*1 genomes differ from that of MoMuLV in the loss of the *Xba*I site at nucleotide 5325. In addition, the *Hin*dIII-*Pst*I segment of the *ts*1 genome has lost the *Xba*I site present at nucleotide 8113 in both of the wt genomes.

The *Hin*dIII-*Pst*I segment of the molecularly cloned infectious DNA of *ts*1-20, another Charon 21A *ts*1 recombinant obtained in a separate cloning experiment, and *ts*7-22 were isolated and digested with the same endonuclease restriction enzymes. The endonuclease restriction map of the *Hin*dIII-*Pst*I genomic fragments of *ts*1-20 and *ts*7-22 cannot be distinguished from that of *ts*1-19 (data not shown).

Results of the endonuclease digests of the *Kpn*I-*Kpn*I genomic sequences of MoMuLV, MoMuLV-TB, and *ts*1 from nucleotide 37–2862 and the *Kpn*I-*Hin*dIII sequences from nucleotide 2863–4894 are shown in Fig. 5A through D. Differences between the genomes were only observed in the *Bam*HI and *Bgl*II digests of the *Kpn*I-*Kpn*I DNA segments and the *Sac*I and *Xho*I digests of the *Kpn*I-*Hin*dIII segments.

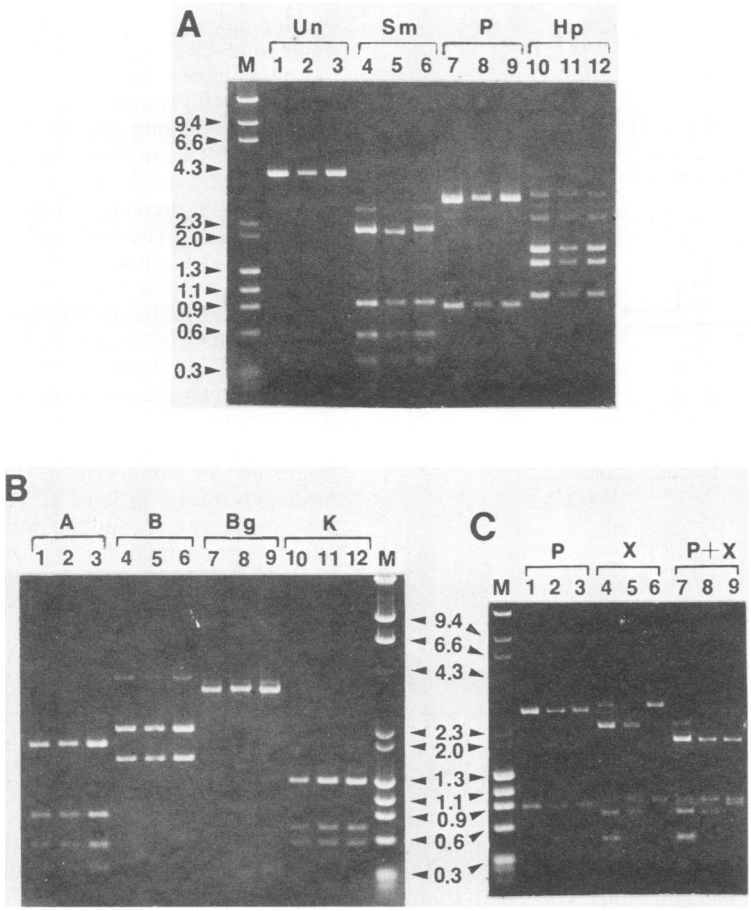

FIG. 4. Endonuclease digests of the *Hin*dIII-*Pst*I restriction fragments from nucleotide 4894-8264 and 1-567 restriction fragments of wt-8.2 (panels A and B: lanes 1, 4, 7, and 10; panel C: lanes 1, 4, and 7); wt-25 (panels A and B: lanes 2, 5, 8, and 11; panel C: lanes 2, 5, and 8); *ts*1-19 (panels A and B: lanes 3, 6, 9, and 12; panel C: lanes 3, 6, and 9). M, *Hin*dIII-restricted fragments of λ and *Hae*III digests of φX174. The endonucleases used were *Sma*I (Sm), *Pvu*II (P), *Hpa*I (Hp), *Ava*I (A), *Bam*HI (B), *Bgl*II (Bg), *Kpn*I (K), and *Xba*I (X). Un, Unrestricted fragment.

Figure 5A, lane 2 shows the expected 1.9- and 1.0-kbp bands on *Bgl*II cleavage of the *Kpn*I-*Kpn*I fragment of MoMuLV at nucleotide 1906. In contrast, the homologous DNA segments of both *ts*1 and MoMuLV-TB were not cleaved by *Bgl*II (lanes 4 and 6).

No *Bam*HI site was found in the *Kpn*I-*Kpn*I DNA segments of MoMuLV (Fig. 5B, lane 2) and MoMuLV-TB (Fig. 5B, lane 4). In contrast, the homologous *ts*1 DNA was cleaved once, generating two fragments of approximately 2.2 and 0.6 kbp (Fig. 5B, lane 6). To locate the *Bam*HI site in the *ts*1 DNA, the *Kpn*I-*Kpn*I *ts*1 DNA segment was digested with both *Bam*HI and *Pst*I. Figure 5D, lane 2, shows the expected ca. 2.1- and 0.5-kbp fragments on *Pst*I digestion (the small 0.2-kbp *Pst*I-*Pst*I fragment is not visible on the ethidium bromide-stained gel). On cleavage with *Bam*HI and *Pst*I, 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 *Bam*HI site at about 0.6 kb from the 3′ end of the *Kpn*I-*Kpn*I *ts*1 DNA segment.

Examination of the nucleotide sequence of MoMuLV at about 0.6 bp upstream from the *Kpn*I site at nucleotide 2863 showed that, beginning at nucleotide 2355, there is a sequence GGACCC and, beginning at nucleotide 2380, there is another sequence GGGTCC (11). A single-base substitution in either sequence will generate a *Bam*HI 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 *Bam*HI site to either of these two sites.

On *Xho*I digestion, the *Kpn*I-*Hin*dIII segment of MoMuLV-TB is cleaved (Fig. 5B, lane 11), which is in contrast to the absence of an *Xho*I site in both MoMuLV (Fig. 5B, lane 9) and *ts*1 (Fig. 5B, lane 13). To locate the *Xho*I site in the MoMuLV-TB DNA, the *Kpn*I-*Hin*dIII DNA segment was doubly digested with *Xho*I and *Sal*I. Restriction with *Sal*I generated the expected two fragments of approximately 1.2 and 0.8 kbp (Fig. 5D, lane 6), since the *Sal*I site is located at about 0.8 kbp from the *Kpn*I site. On restriction with *Xho*I and *Sal*I 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 *Xho*I site is located about 0.4 kbp from the *Kpn*I site. The other λ recombinant DNAs of MoMuLV-TB λwt-14 and λwt-17 were also restricted with *Xho*I, and both were found to possess the additional *Xho*I site in the *Kpn*I-*Hin*dIII segment of the *pol* gene. Examination of the nucleotide sequence of MoMuLV (11) at about 0.4 kbp from

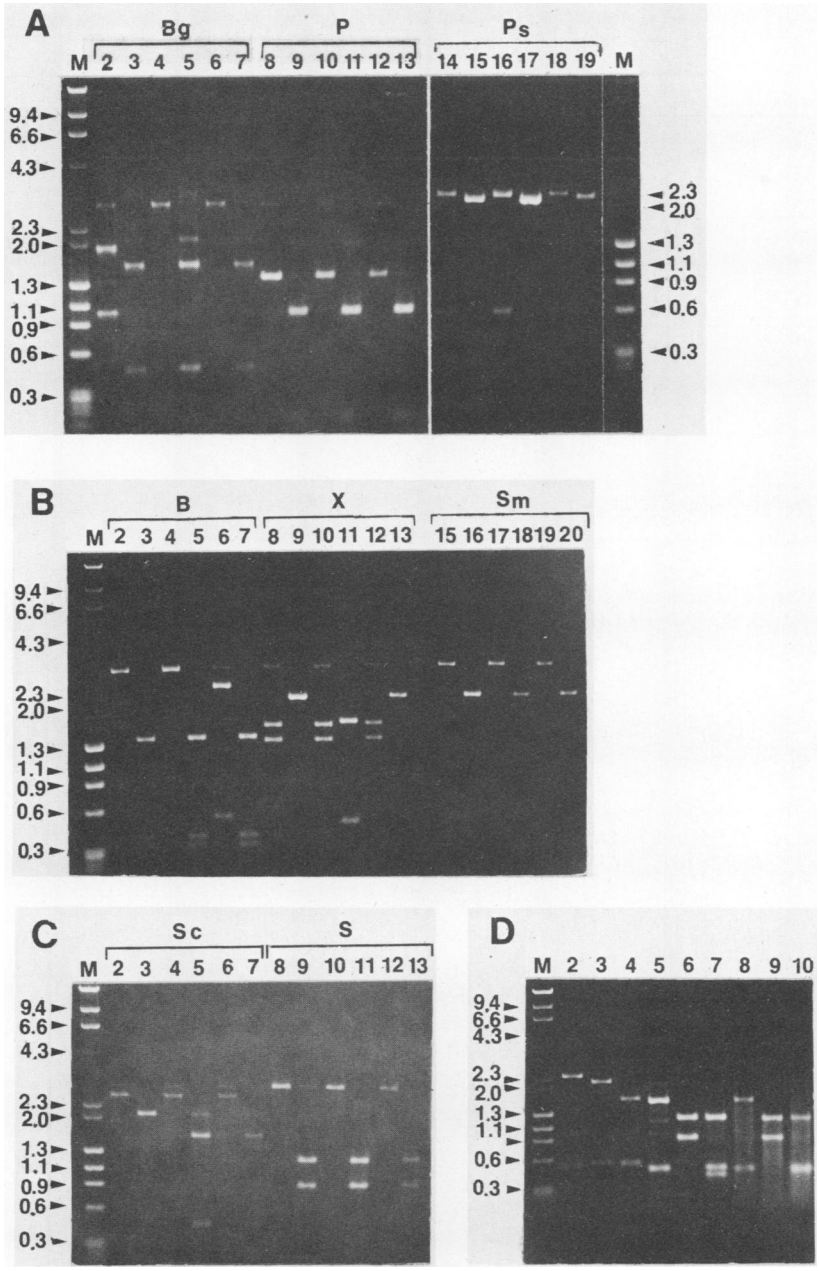

FIG. 5. Endonuclease digests of the *Kpn*I-*Kpn*I sequence from nucleotide 36-2862 of wt-8.2, wt-25, and *ts*1-19 (panels A, B, and C: even-numbered lanes) and the *Kpn*I-*Hin*dIII sequence from nucleotide 2863-4894 of wt-8.2, wt-25, and *ts*1-19 (panels A, B, and C: odd-numbered lanes). M, *Hin*dIII-restricted fragments of λ and *Hae*III digests of φX174. The endonucleases used were *Bgl*II (Bg), *Pvu*II (P), *Pst*I (Ps), *Bam*HI (B), *Xho*I (Xh), *Sma*I (Sm), *Sac*I (Sc), and *Sal*I (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 2, 3, 8, and 9. 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. *ts*1-19 DNA: panel A, lanes 6, 7, 12, 13, 18, 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: *Kpn*I-*Kpn*I DNA sequence of *ts*1-19 restricted with *Bam*HI (lane 2), *Pst*I (lane 3), and *Bam*HI and *Pst*I (lane 4); lanes 5 through 10: *Kpn*I-*Hin*dIII DNA sequence of wt-25 restricted with *Xho*I (lane 5), *Sal*I (lane 6), *Xho*I and *Sal*I (lane 7), *Sac*I (lane 8), *Sal*I (lane 9), and *Sac*I and *Sal*I (lane 10). M, *Hin*dIII restriction fragments of λ and *Hae*III restriction fragments of φX174.

the *Kpn*I site showed that, beginning at nucleotide 3316 and 3345, there is a sequence CTCGGG which is an *Ava*I recognition site. Substituting the G residue at nucleotide 3320 or 3349 with an A residue will generate an *Xho*I recognition site while retaining the *Ava*I recognition site. We have therefore tentatively assigned the *Xho*I site to one of the *Ava*I 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 *Kpn*I-*Kpn*I and *Kpn*I-*Hin*dIII segments of the molecularly cloned DNA of *ts*1-20 and *ts*7-22 were isolated and similarly endonuclease restriction mapped. The infectious

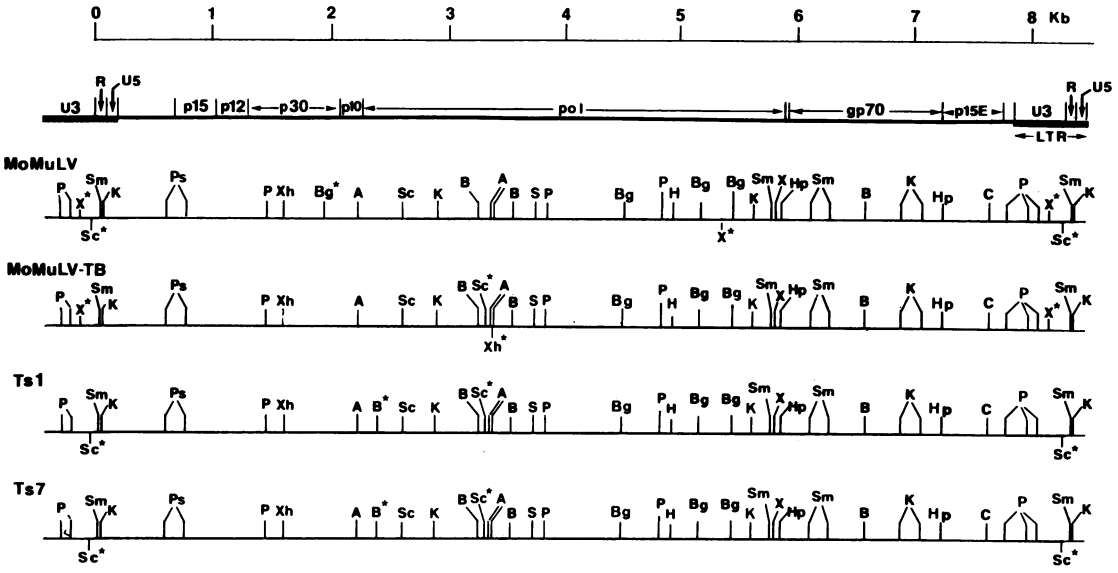

FIG. 6. Schematic presentation of the restriction maps of standard MoMuLV (wt-8.2), MoMuLV-TB (wt-25), *ts*1 (*ts*1-19), and *ts*7 (*ts*7-22). The endonucleases used were *Ava*I (A), *Bam*HI (B), *Bgl*II (Bg), *Cla*I (C), *Hin*dIII (H), *Hpa*I (Hp), *Kpn*I (K), *Pvu*II (P), *Pst*I (Ps), *Sal*I (S), *Sac*I (Sc), *Sma*I (Sm), *Xba*I (X), *Xho*I (Xh). *, Restriction sites which differ among MoMuLV, MoMuLV-TB, *ts*1, and *ts*7 DNAs.

DNA of *ts*1-20 and *ts*7-22 could not be distinguished from that of *ts*1-19 at this level of endonuclease restriction analysis.

The restriction maps of the molecularly cloned DNAs of wt-8.2, wt-25, *ts*1-19, and *ts*7-22 of standard MoMuLV, MoMuLV-TB, *ts*1, and *ts*7, respectively, are schematically presented in Fig. 6.

DISCUSSION

To identify the change(s) in the genome which confers on *ts*1 and *ts*7 the temperature sensitivity, inefficiency in the processing of $Pr80^{env}$, and the ability to induce hind-limb paralysis, we have molecularly cloned *ts*1, *ts*7, and MoMuLV-TB. The infectious viruses obtained on transfection of the molecularly cloned *ts*1-19, *ts*7-22, 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 *ts*1 and *ts*7 has the same electrophoretic mobility as the p30 of MoMuLV-TB (17). The *Bgl*II site present at nucleotide 1905 at the 5′ end of the p30 coding sequence of MoMuLV is absent in the MoMuLV-TB, *ts*1, and *ts*7 genomes. This finding clearly shows that this segment of the genome is mutated. However, whether the mutation at the *Bgl*II site in the MoMuLV-TB, *ts*1, and *ts*7 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 injected 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 *ts*1 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 *Sac*I site in U3 and the loss of the *Xba*I 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 *ts*1 and *ts*7 obtained with 11 enzymes were similar. This is not altogether unexpected. Despite the fact that *ts*1 and *ts*7 were isolated on separate occasions, they share certain common characteristics. However, they are distinguishable in that mice infected with *ts*7 need a slightly longer latent period before developing paralysis than those infected with *ts*1 (Fig. 3) and that the virions of *ts*7 are more heat labile than those of *ts*1 (Fig. 1). A more detailed analysis of these genomes is now in progress to identify the base change(s) which confers on *ts*7 virions heat lability and the attenuated ability to induce paralysis.

*ts*1 and *ts*7 differ from MoMuLV in the loss or acquisition of four different restriction sites, whereas *ts*1 and *ts*7 differ from MoMuLV-TB in the loss or acquisition of three different restriction sites. However, only two changes are peculiar to *ts*1 and *ts*7 and not shared with either of the wt genomes. These are the loss of the *Xba*I site at nucleotide 8113 and the presence of an additional *Bam*HI 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 *Hin*dIII-*Bam*HI genomic sequence of *ts*1 from nucleotide 4894 to 6538 was found to determine the temperature sensitivity, processing of $Pr80^{env}$, and possibly the ability to induce paralysis. Studies are now in

progress to determine by exchanging genomic fragments within the *Hin*dIII-*Bam*HI sequence and nucleotide sequencing whether the *ts* function, the inefficiency in the processing of $Pr80^{env}$, and the ability to induce paralysis result from a single mutation or multiple mutations.

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