Genetic Studies of the Ploidy of Moloney Murine Leukemia Virus

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An assay for Moloney murine leukemia virus was developed that made use of the production of morphologically altered foci in nonproducer mouse cells (15F) carrying murine sarcoma virus. Wild-type (*wt*) virus gave a ratio of titers at 39°C/34°C = 1.05 ± 0.45 (standard deviation; $n = 20$). A spontaneous, thermosensitive (*ts*) mutant of Moloney murine leukemia virus, *ts3*, defective in a late viral function, gave 39°C/34°C = 0. A murine cell line (TB) was mixedly infected with *ts3* and *wt* (multiplicities of infection, 7.8:4.3), cloned after infection, and shown to be infected by both viruses. At 34°C it produced *wt*, *ts*, and particles of mixed parentage. The heterozygotes (*hz*) had ratios of assays 39°C/34°C = 0.06 to 0.84 (mean, 0.36). To eliminate possible interference by multiploid particles with determination of the proportions of the three types of particles, the virus produced by the mixedly infected, cloned cell line at 34°C was distributed by velocity sedimentation in a sucrose gradient, and virus was picked from the lightest part of the gradient. The proportions of *ts*, *wt*, and *hz* were 0.27, 0.26, and 0.47. Those particles identified as *hz* segregated *ts*, *wt*, and *hz* in the proportions 0.24, 0.27, and 0.49, respectively. These values were not significantly different from those predicted from a diploid model of the genome.

In a recent study (15) the weight-average molecular weight for native and subunit RNA species of Moloney murine leukemia virus (M-MLV) was measured by using sedimentation equilibrium ultracentrifugation. This method, which yields molecular weights independent of conformation of the macromolecules, gave a molecular weight of 7.2×10^6 for the native RNA and 3.4×10^6 for the subunit species. These data led to the conclusion that the RNA of M-MLV consists of two subunits.

Electron microscopic studies have shown that the RNA of several different type C particle viruses consists of dimers linked at their 5' ends by a secondary structure. Published reports are for RD-114 (11), an endogenous xenotropic baboon virus, woolly monkey virus (12), and Friend virus (6). Although M-MLV has not been included, it is unlikely to be different.

Measurements of the genetic complexity of the RNA of M-MLV, based on an analysis of unique RNase-T₁-resistant oligonucleotides, gave a value of 3.5×10^6 daltons, which is in close agreement with the size of the subunit measured by physical means (3). These studies thus indicated that the genome is diploid. On the other hand, earlier data on the complexity of the RNA of M-MLV obtained by hybridizing viral RNA with complementary DNA and

measuring the kinetics of hybridization, as compared with the rate of hybridization of poliovirus RNA with poliovirus-specific DNA (7), gave a value of 9×10^6 . This value would indicate that the genome is haploid.

Because of the nature of the assumptions made in these molecular biological studies and the fact that they are of viral stocks that always contain a minority of viral particles having biological activity, it is important to provide confirmatory genetic evidence for diploidy. A genetic test for diploidy might be made by mixedly infecting a cell with wild-type (*wt*) M-MLV and a thermosensitive (*ts*) mutant of the same virus. Random reassortment of genomic subunits should yield *ts*, heterozygotes (*hz*), and *wt* in proportions given by the terms of the expansion of $(x + y)^2$, respectively (9, 19). A haploid model would yield *ts* and *wt* only, and a triploid model should give *ts*, *hz*, and *wt* in proportion to the terms of the expansion of $(x + y)^3$.

Possible complications would be expected to include inefficiency of genetic mixing or selection of one sort of progeny during growth of the virus. More probably, the production of multiploid viral particles might obscure the results. Particles having two cores have been observed occasionally by electron microscopy and were

much larger than unit particles (14). Particles having two cores and twice the volume of a unit particle together with larger particles with multiple cores have been estimated by electron microscopy to occur in M-MLV, with a frequency of approximately 8% (P. H. Yuen and P. K. Y. Wong, personal communication). We attempted to separate such particles from consideration by subjecting the virus to velocity sedimentation, taking only the lightest virus for analysis, as suggested by Simon (16).

We developed a new, direct focus assay for MLV. The assay is based on the use of cells described by Ball et al. (1, Table 8) as M15. M15 and several clones like it were isolated from a mouse cell line infected with a preparation of Moloney murine sarcoma virus (MSV). These cloned cell lines did not appear to be transformed, but formed flat monolayers. M15 cells were subcloned, and one subclone, 15F, has been used for the work reported here. These 15F cells do not produce any viral particles detectable by [³H]uridine labeling (J. K. Ball, personal communication) or any detectable by reverse transcriptase assays (McCarter, unpublished data) and do not express any more gp71 (AKR) than do uninfected TB cells (D. P. Bolognesi, personal communication). When 15F cells are superinfected with MLV, both MSV and MLV are produced and the cells become morphologically transformed. It is this property that has been made the basis for the direct focus assay. These cells differ from the S⁺L⁻ cells of Bassin et al. (2) in that 15F cells produce no particles, and they differ from the cell line reported by Hackett and Sylvester (8) in that 15F cells contain MSV. These 15F cells have been carried over a period of more than 2 years and 90 serial passages without losing their flat morphology or the ability to respond, quantitatively, in the same way to superinfection with MLV. The properties of 15F cells are currently under investigation.

MATERIALS AND METHODS

Cells. The origins of TB and 15F cells have been described (1). Cells were grown in Eagle minimum essential medium supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co.), 50 U of penicillin per ml, and 50 µg of streptomycin per ml. In some instances, as noted below, 50 µg of gentamicin per ml was used as well (Schering Corp.). Other conditions of culture are given below. XC cells were obtained from J. Hartley and carried as described by Klement et al. (10).

Viruses. The origin of the parental *wt* M-MLV and the isolation of *ts* mutants of M-MLV, called *ts1* and *ts3*, have been described (23). M-MLV *ts1* is *ts* in an early function, and *ts3* is involved in a very late function associated with budding from the cell mem-

brane (21, 22). Stocks of the *ts* mutants and the *wt* virus were grown as described (23) or as given below.

Assays for MLV. The XC infectious centers assay was performed as described by Wong et al. (23).

The direct focus assay was performed as follows. 15F cells were grown in T75 flasks (Falcon Plastics) at an initial density of 1×10^6 cells/bottle in 20 ml of complete medium at 37°C in an incubator containing 5% CO₂ in humidified air. When they approached confluence (3 to 4 days), the cells were removed from the flasks with trypsin (0.25% in isotonic saline citrate), centrifuged, and resuspended in complete medium to a concentration of 1×10^6 cells/ml.

Virus was diluted in medium lacking serum and containing 25 µg of DEAE-dextran (Pharmacia) per ml (17). Diluted virus (1.0 ml) was mixed with 1.0 ml of cell suspension (1×10^6 cells) in sterile, disposable plastic tubes (12 by 75 mm; Falcon Plastics) and shaken at 37°C for 20 min. The suspension was then diluted with complete medium, and 3 ml containing 1×10^5 cells was transferred to 35-mm dishes (Lux Scientific Corp., Thousand Oaks, Calif.). Cultures infected with *ts* mutants were placed at 34°C, the permissive temperature for focus formation, or at 39°C, the nonpermissive temperature for these *ts* mutants. Maximal focus formation required 5 days at 34°C and 3 days at 39°C. If the medium was to be replaced with complete medium containing 0.7% agar, this was done after the cells had attached. Focus formation under agar then took 6 days at 34°C and 4 days at 39°C. The foci consisted of plaques, 0.5 to 1 mm in diameter, formed by the rounding up of cells within the plaque. For a quantitative assay, the foci were counted alive, using an inverted microscope, or cells were fixed with 10% Formol-saline and stained with 0.1% aqueous methylene blue.

Foci were picked by marking all the foci and selecting only those that were far enough from the others to ensure only a minimal chance of cross-contamination. Cells from the focus were placed in 3 ml of complete medium in a 35-mm dish. The dish was then incubated at 34°C, and the medium was collected for assay.

Reverse transcriptase assay. Reaction mixtures contained 0.05 M Tris-hydrochloride (pH 8.0), 0.06 M KCl, 2×10^{-4} M manganese acetate, 0.002 M dithiothreitol, 0.01 absorbance at 260 nm unit of poly(rA):oligo(dT)₁₂₋₁₈, 2×10^{-5} M [³H]TTP (5,000 cpm/pmol; New England Nuclear Corp.), plus the virus in 0.1% Triton X-100 in 0.05 M Tris-hydrochloride (pH 8.0) to a final volume of 0.1 ml. The mixture was incubated at 37°C for 60 min, and then 0.5 ml of ice water and 0.3 ml of 33% trichloroacetic acid saturated with sodium pyrophosphate was added. The precipitated material was collected on a membrane filter (0.45 µm; Millipore Corp.), washed with 5% trichloroacetic acid-50% ethanol, and dried. The radioactivity was measured by using a liquid scintillation spectrometer.

Electron microscopy. Virus preparations were examined by a negative-staining technique (4).

Centrifugation. Sterile, linear 10 to 30% sucrose gradients were prepared in complete medium, using an ISCO model 570 gradient former (Instrument

Specialties Co., Lincoln, Neb.). The two solutions used to prepare the gradient were sterilized by filtration, and the syringes and tubing of the gradient former were washed first with 70% ethanol and then with sterile distilled water. A 12-ml amount of gradient was placed on top of a cushion of 0.3 ml of 65% sucrose in a cellulose nitrate centrifuge tube (0.56 by 3.75 inches [ca. 1.4 by 9.5 cm]; Beckman). After formation of the gradient, 1 ml of medium in which the infected cells had grown was placed on top of the gradient, and the tube was centrifuged at 20,000 rpm for 2 h at 4°C in a Spinco SW40 rotor in an L2-65B ultracentrifuge (Beckman). At the end of that time, the tube was removed from the rotor and 0.2-ml portions were withdrawn from the top of the tube, using an ISCO density gradient fractionator model 640 (Instruments Specialties Co., Lincoln, Nebr.). Fractions were assayed for reverse transcriptase activity directly by taking 20- μ l portions from the tubes containing the fractions. Assays for infectious virus were performed by diluting the fractions with sterile medium containing 25 μ g of DEAE-dextran per ml and lacking serum (17). The diluted fractions were assayed by using 15F cells in the direct focus assay. In these assays the medium always contained 50 μ g of gentamicin (Schering Corp.) per ml in addition to penicillin and streptomycin.

RESULTS

Characteristics of the direct focus assay. Table 1 shows that the direct focus assay was "one-hit" over the useful range of dilution for *wt* M-MLV. The ratio of titers obtained for *wt* M-MLV at 39 and 34°C was 1.05 ± 0.45 (standard deviation [SD]; $n = 20$).

When used to assay the mutants M-MLV *ts1* and M-MLV *ts3*, it was apparent (Table 1) that the results were "one-hit" at 34°C, but not at 39°C. Multiplicity-dependent leakiness has

been described before for these mutants (21).

To test whether foci initiated by *ts* mutants could be distinguished from those initiated by *wt* virus, 15F cells were infected with a mixture of M-MLV *ts3* and *wt*. The preparations, which had been grown and assayed separately, were diluted and mixed prior to infection of the cells and growth at 34°C so that a predetermined number of foci of each type could be produced. Of 35 foci picked, 15 were *ts* (expected 19) and 20 were *wt* (expected 16). In each case the values found were not significantly different from the expected values (*ts3* = $0.9 > P > 0.8$; *wt* = $0.95 > P > 0.9$); i.e., when foci produced by a mixture of *ts3* and *wt* were examined, those produced by *wt* could be distinguished from those produced by *ts3*. For *ts3* alone, of 46 foci, 42 had a ratio of titers 39°C/34°C = 0, and the remaining foci had titer ratios of 0.002, 0.004, 0.005, and 0.13.

Isolation of mixedly infected cells. To ensure that TB cells were mixedly infected with M-MLV *ts3* and M-MLV *wt*, cloned cells were isolated after exposure to a mixture of *ts3* and *wt*. To do this, the multiplicities of infection (MOIs) for each infecting virus were determined as accurately as possible, using the XC infectious center assay, and TB cells were infected in suspension by shaking 2 ml containing 2×10^6 cells with 2 ml of mixed, diluted virus for 20 min at 37°C. After infection of the TB cells, 1×10^6 cells were transferred to a T75 flask (Falcon Plastics) in 20 ml of medium and grown for 5 days at 34°C. The medium was replaced, and a 24-h harvest was collected (bulk harvest). At the same time, a portion of the infected cell suspension containing 200 cells in 4

TABLE 1. Titration of M-MLV as a function of dilution and nature of virus at different temperatures

Virus	Assay	Dilution	Titers (U/ml)		
			37°C	39°C	34°C
<i>wt</i>	XC		1.8×10^8		
		1:20,000	4.0×10^{7a}		
		1:40,000	2.9×10^7		
		1:80,000	3.4×10^7		
		1:200,000	3.3×10^7		
M-MLV <i>ts1</i>	15F	1:1,000,000	2.8×10^7		
		1:5,000		2×10^6	TMTC ^b
		1:10,000		3×10^5	TMTC
		1:20,000		0	1.2×10^8
		1:40,000		0	1.6×10^8
M-MLV <i>ts3</i>	15F	1:80,000		0	1.4×10^8
		1:5,000		5×10^5	TMTC
		1:10,000		0	TMTC
		1:20,000		0	2.0×10^8
		1:40,000		0	2.4×10^8

^a Ratio of titers for *wt* 39°C/34°C = 1.05×0.45 (SD; $n = 20$).

^b TMTC, Too many to count.

ml of medium was transferred to 60-mm dishes. After the cells had attached, the medium was removed and replaced with medium containing 0.7% agar. The dishes were incubated at 34°C for 7 days and then, from 10 individual, well-spaced colonies for each set of MOIs, cells were transferred to 35-mm dishes containing 3 ml of medium and incubated at 34°C. These cells were cultured, transferred, and grown to confluence in T25 flasks (Falcon Plastics) at 34°C. The medium in which the cells had grown was collected for assay. The cells were transferred as they reached confluence to new T25 flasks, and the medium in which they had grown was assayed each time they had grown to confluence. Table 2 shows the results obtained by assay of the so-called "bulk harvest" derived from the growth of 1×10^6 cells infected at each set of ratios of MOIs of *ts3* and *wt* and those averaged for 10 colonies examined at passage 4 for the same sets of MOIs.

First, it was found (Table 2) that, for each set of MOIs, all 10 colonies tested were infected. Second, the ratio of titers 39°C/34°C averaged for the 10 colonies of a set was not significantly different from the value for the corresponding bulk harvest. Third, the value of the ratio of titers 39°C/34°C appeared to be correlated with the ratio of MOIs used to establish the infection.

In a preliminary experiment, one of the cloned cell cultures was selected for the set *wt:ts3* = 4.3:31 at passage 4. The virus produced by this clone had a ratio of titers 39°C/34°C = 0.17. It was diluted, filtered through a membrane filter (0.45 μ m; Millipore Corp.), and used to infect 15F cells for the production of foci at 34°C under agar, as described above. Twenty-one foci were picked and grown up, and the virus was assayed at 34 and 39°C. The virus grown up from each focus was then used to

infect 15F cells so that new foci would result, and, for each of the 21 foci, 10 new foci produced at 34°C were picked, grown up, and assayed. Of the 21 foci picked, 4 had 39°C/34°C ratios = 0, and subfoci were also *ts*. These four foci were, therefore, unambiguously *ts*. For a ratio 39°C/34°C = 0.17, a haploid model predicts the proportion *ts* = 0.83; i.e., there should have been 17 or 18 foci that were *ts*. The observed value is significantly different ($P = 0.01$) (13) from the value expected of a haploid genome. Of the remaining 17 foci, 10 appeared to be *wt* on the basis of the ratio of titers 39°C/34°C and subfoci, and 7, on the basis of what we learned later (see Table 4) and from the recovery of *ts* virus among the subfoci of 2 of them, were *hz*. According to Hardy-Weinberg equilibrium (9, 19), a value of $4/21 = ts$ predicts 7/21 for *wt* and 10/21 for *hz*. The numbers found are not significantly different from these predicted values: according to Mainland et al. (13), significance at the 5% level would require 7/21 to be contrasted with a minimum of 14/21. The observed values are within that range.

In a more extensive experiment, we decided to follow the suggestion of Simon (16) to employ velocity sedimentation of the virus in a sucrose gradient (5) as a way to get rid of possible aggregates and multiploid particles. Details of the procedure have been described above.

For this experiment, we used a fresh, 24-h viral harvest taken on day 6 from TB cells infected with virus from one of the cloned cell cultures of the set *wt:ts3* = 4.3:7.8 at passage 4. The ratio of titers 39°C/34°C for this stock was 0.5. The medium in which the infected cells had been grown was centrifuged to remove any cells and diluted 1:2 with complete medium. This preparation was not filtered or frozen prior to placing 1 ml on the top of the gradient. The results of the experiment that provided the data of Table 4 are given in Table 3. Infectious virus and reverse transcriptase activities were found in the same peak. The peak represented virus that was sedimenting rather than virus at an equilibrium position in the gradient because, by centrifuging for a longer time at the same speed, the peak was found farther down the tube (data not shown). Electron microscopy of the fractions revealed no clumped particles throughout the peak. Only single, type C particles were seen. There were single particles of slightly larger diameter at the bottom of the gradient than at the top, but quantitation was not attempted because the presence of sucrose made the measurements uncertain. Virus taken from fraction 60, near the bottom of the tube, when regrown and assayed by velocity

TABLE 2. Mixed infection of TB cells with *M-MLV ts3* and *M-MLV wt* at different MOIs^a

MOI of <i>wt/ts3</i>	Bulk harvest		Clones (39°C/ 34°C ratio)
	Titer (FFU ^b / ml)	39°/34°C ratio	
4.3:31	6.6×10^7	0.22 ± 0.09^c	0.21 ± 0.08
4.3:7.8	5.6×10^7	0.54 ± 0.06	0.43 ± 0.13
17.1:7.8	6×10^7	0.50 ± 0.04	0.77 ± 0.12
		$n = 4$ each	$n = 10$ each

^a Titer at 34°C and ratio of titers 39°C/34°C as a function of MOI for bulk harvest and the ratio 39°C/34°C for cloned, mixedly infected cells.

^b FFU, Focus-forming unit.

^c Mean $\pm$ SD.

TABLE 3. Distribution of virus by sedimentation velocity in 10 to 30% sucrose gradients at 20,000 rpm for 2 h at 4°C in a Spinco SW40 rotor

Fraction no.	Assay (FFU/ml $\times 10^{-4}$) ^a		Reverse transcriptase (cpm/ml) ^b
	39°C	34°C	
21	2.1	3.3	1,790
24	5.9	7.8	6,040
27	24	55	9,735
30	92	132	18,750
33	129	254	24,195
36	138	170	31,735
39	18.7	23.2	20,730
42	16.3	22.4	12,720
45	10.2	22	7,045
48	5.2	11.2	5,485
51	2.2	4.5	3,965
54	9.4	7.4	1,215
57	8.4	12.3	ND ^c
60	12	11.9	ND

^a Titers of fractions (0.2 ml) assayed (15F) at 34 and 39°C. FFU, Focus-forming units.

^b Corrected for background.

^c ND, Not determined.

sedimentation, regenerated the same pattern as seen in Table 3, with a peak in the same tube (fraction 36).

Virus was taken from the trailing edge of the sedimenting peak (fraction 21) and used to infect 15F cells so that, under agar, there was an average of 9 foci/35-mm dish at 34°C. A total of 55 foci were tested, and many of these were subcloned by the production of new foci in 15F cells. The data are shown in Table 4.

The data are arranged in order of increasing value of the ratio of titers obtained at 39°C divided by the titer obtained at 34°C. Assignment of a particular focus to the categories *ts*, *wt*, and *hz* depended upon three criteria: (i) the value of the 39°C/34°C ratio, (ii) the examination of subclones (foci) of the viral progeny from the initial foci (numbered 1 to 55 in Table 4), and (iii) the morphology of foci produced at 39°C. Foci produced by *wt* virus at 39°C were as large in diameter as those produced at 34°C, but foci produced at 39°C by the progeny of an *hz* consisted of a mixture of large and small foci. The latter were difficult to count.

An examination of Table 4 shows that the first 15 foci were *ts*. The 16th foci was probably also *ts*. There followed 24 or 25 (no. 40 was not subcloned) that were *hz*. The remaining 14 were judged to be *wt*. The proportions were, therefore, *ts* = 0.27 to 0.29; *hz* = 0.44 to 0.51, and *wt* = 0.25 to 0.27.

In determining whether these observed values agreed with predicted values, we noted (Table 4) that the 39°C/34°C ratios for foci produced

by heterozygotes lay between 0.06 and 0.84 (mean, 0.36). The *ts* character is, therefore, not completely dominated by the presence of a *wt* subunit. For *ts* codominant with *wt* in a diploid genome, the predictions from Hardy-Weinberg equilibrium (9, 19) for a mixture of viruses having 39°C/34°C = 0.5 and for a mean 39°C/34°C for *hz* = 0.36 are: *ts* = 0.18, *hz* = 0.49, and *wt* = 0.34. The observed and expected frequencies do not differ significantly (*P* = 0.8).

A further test of the hypothesis could be made by assigning all the subfoci produced by *hz* to the categories *ts*, *hz*, and *wt*, using as criteria the values for 39°C/34°C derived from Table 4; i.e., *ts* = 0 to 0.06, *hz* = 0.07 to 0.84, and *wt* = 0.85 to 1.26. When all subfoci that were thus classified as *ts* were added together, they amounted to 58 of a total of 241, i.e., 0.24. The frequencies of *hz* and *wt* were 119/241 = 0.49 and 64/241 = 0.27, respectively. These values are not significantly different from those predicted by a diploid model (0.8 < *P* < 0.9). They are not consistent with a haploid model, which predicts no *hz*, and the values found agree better with a diploid model than with a triploid model (prediction for 39°C/34°C = 0.5; *ts* = 0.02; *hz* = 0.56; *wt* = 0.42; 0.1 < *P* < 0.2).

DISCUSSION

Molecular and electron microscopic studies (3, 6, 11, 12, 15) indicate that the RNA of type C particle viruses consists of two subunits that are identical. This conclusion would be greatly strengthened if it could be shown by genetic studies that the genome is diploid.

We developed procedures for such genetic studies, involving a direct focus assay for MLV in cells (15F) that carry MSV and undergo morphological transformation when superinfected with MLVs. Although MSV is rescued from such cells, there is no evidence that it affects assays for MLV or that it complements the *ts* function of the mutant that was used in this work. The ratio of titers 39°C/34°C in the focus assay of *ts3* on 15F cells was 0, and for virus grown up from 42 foci produced by *ts3* in 15F cells at 34°C it was also 0. Four other foci with 39°C/34°C ratios = 0.002, 0.004, 0.005, and 0.12 produced some foci at 39°C. The most probable source of these was reversion to *wt* during the multiple cycles of infection that must have occurred by a spread of virus during the production of a focus. *wt* virus in the 15F focus assay produces as many foci as 39°C as at 34°C (39°C/34°C = 1.05 $\pm$ 0.45 [SD; *n* = 20]).

It was critical to distinguish *hz*. These were identified by means of the ratio 39°C/34°C,

TABLE 4. *Analysis of foci and subfoci of progeny virus of mixedly infected TB cells^a*

Focus no.	39°C/34°C assay	39°C/34°C ratio	Subfoci			Genotype
			<i>ts</i>	<i>hz</i>	<i>wt</i>	
1	0/36	0	10	0	0	<i>ts</i>
2	0/62	0				<i>ts</i>
3	0/40	0				<i>ts</i>
4	0/50	0				<i>ts</i>
5	0/38	0				<i>ts</i>
6	1/150	0.007				<i>ts</i>
7	1/120	0.008				<i>ts</i>
8	1/76	0.013				<i>ts</i>
9	1/72	0.014	12	0	0	<i>ts</i>
10	1/44	0.02				<i>ts</i>
11	2/65	0.03	9	1	0	<i>ts</i>
12	1/25	0.04	11	1	0	<i>ts</i>
13	2/38	0.05	12	0	0	<i>ts</i>
14	12/187	0.06	11	0	0	<i>ts</i>
15	2/35	0.06	10	0	0	<i>ts</i>
16	3/48	0.06	7	0	1	<i>hz</i> (?)
17	9/142	0.06	6	2	2	<i>hz</i>
18	16/200	0.08	1	9	0	<i>hz</i>
19	6/71	0.08	8	2	0	<i>hz</i>
20	7/80	0.09	1	1	8	<i>hz</i>
21	18/200	0.09	2	7	1	<i>hz</i>
22	2/18	0.11	5	5	0	<i>hz</i>
23	5/39	0.13	0	7	1	<i>hz</i>
24	35/250	0.14	5	1	3	<i>hz</i>
25	23/151	0.15	0	9	1	<i>hz</i>
26	5/32	0.15	2	4	3	<i>hz</i>
27	71/320	0.22	2	8	0	<i>hz</i>
28	10/41	0.24	3	5	2	<i>hz</i>
29	88/300	0.29	7	3	0	<i>hz</i>
30	20/63	0.32	2	2	6	<i>hz</i>
31	18/55	0.33	0	8	2	<i>hz</i>
32	22/60	0.37	0	6	3	<i>hz</i>
33	150/320	0.47	1	2	7	<i>hz</i>
34	102/200	0.51	1	7	2	<i>hz</i>
35	140/214	0.65	0	9	1	<i>hz</i>
36	16/24	0.67	0	6	4	<i>hz</i>
37	14/19	0.74	3	3	4	<i>hz</i>
38	6/8	0.75	1	9	0	<i>hz</i>
39	80/105	0.76	0	1	9	<i>hz</i>
40	125/162	0.77				<i>hz</i> (?)
41	16/19	0.84	1	3	4	<i>hz</i>
42	150/175	0.85				<i>wt</i>
43	82/96	0.85				<i>wt</i>
44	300/300	1.0				<i>wt</i>
45	30/30	1.0				<i>wt</i>
46	200/200	1.0				<i>wt</i>
47	150/150	1.0				<i>wt</i>
48	400/400	1.0	0	0	10	<i>wt</i>
49	400/400	1.0	0	0	10	<i>wt</i>
50	60/61	1.0				<i>wt</i>
51	36/35	1.02	0	0	10	<i>wt</i>
52	105/100	1.05				<i>wt</i>
53	42/40	1.05				<i>wt</i>
54	49/46	1.06				<i>wt</i>
55	180/142	1.26				<i>wt</i>

^a Foci were picked from 15F cells infected with virus from fraction 21 (Table 3).

which for those identified as *hz* lay between 0.06 and 0.84, and by the segregation of *ts*, *wt*, and *hz* virus by subcloning. For the classification of subfoci in Table 4, the only criterion was the 39°C/34°C ratio. Had the range of values used been 0.08 to 0.85, the numbers obtained would have been substantially the same.

The results presented here are consistent with those predicted from a diploid model of the genome of M-MLV and provide biological confirmation of results obtained by physical and molecular approaches (3, 6, 11, 12, 15); i.e., the subunits contain the same genetic information, and there are probably two of them.

The efficiency with which subunits of *ts3* and *wt* were mixed under permissive conditions and the persistence of *hz* into the subfoci (Table 4) agree with predictions made by Vogt (18) that, for a simple polyploid genome, *hz* would be expected to persist indefinitely unless conditions should select for only one genome.

One of the consequences of the present findings is that mixed infection would be expected by complementing *ts* mutants to produce *hz* that might mimic *wt* recombinants. It is now necessary to reexamine the evidence that genuine *wt* recombinants are produced, at least in the murine system (20). A further consequence is that there cannot be sufficient assurance that one has isolated a homozygous leukemia virus by isolating the progeny from a single plaque or by a single limiting dilution, or even by isolating several consecutive plaques of limiting dilutions.

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