JOURNAL OF VIROLOGY, Oct. 1974, p. 918-923
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Vol. 14, No. 4
Printed in U.S.A.

Temperature-Sensitive Mutants of Murine Leukemia Virus

IV. Further Physiological Characterization and Evidence for Genetic Recombination

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Received for publication 21 May 1974

Several temperature-sensitive mutants of the Rauscher strain of murine leukemia virus representing three distinct physiological groups have been further characterized. Genetic recombination between different pairs of these mutants has been demonstrated. Several representative genetic recombinants were isolated and shown to replicate equally well at the permissive (31 C) and nonpermissive (38 C) temperatures and to have serological characteristics of the wild-type parental virus. Alternative models for the mechanisms involved in recombination between type C RNA viruses are discussed.

Conditional lethal mutants of murine leukemia virus (MuLV) have been isolated in an attempt to elucidate the genetic functions involved in replication of mammalian type C RNA viruses (17, 18, 20, 30, 31). Temperature-sensitive (ts) MuLV mutants isolated to date in our laboratory include nine mutants of the Kirsten and twelve of the Rauscher (R-) strains of MuLV. The majority of these mutants have been shown to be defective in replication steps subsequent to absorption and penetration (17, 18). In addition, many of the mutants have been classified into groups based upon their physiological defects in replication at the nonpermissive temperature (17, 18).

Recent evidence has indicated that both avian (12, 26, 27) and mammalian (19, 30) type C RNA viruses can undergo genetic recombination. Whether this mechanism involves subunit reassortment or classic genetic recombination is not yet clear (27). We have attempted to detect and quantitate genetic recombination between several different physiologically characterized ts mutants of R-MuLV.

MATERIALS AND METHODS

Cell culture. Cells were grown in Dulbecco modification of Eagle medium supplemented with 10% calf serum (Colorado Serum Co., Denver, Colo.) in plastic petri dishes (60 by 15 mm; Falcon Plastics, Los Angeles, Calif.). Cells used included a continuous mouse line, NIH/3T3 (11), a rat cell line, NRK (5), and a human rhabdomyosarcoma line, A673 (9).

Viruses. Wild-type R-MuLV, six representative R-MuLV ts mutants, ts 17, ts 19, ts 25, ts 26, ts 28, and ts 29 (17), and an endogenous type C virus of NIH Swiss mouse cells have been described (13; J. R. Stephenson, S. A. Aaronson, P. Arnstein, R. J. Huebner, and S. R. Tronick, Virology, in press).

Virus assays. Type C virus-associated RNA-dependent DNA polymerase activity in tissue culture fluids of infected cells was assayed as previously described (17). An antibody which specifically inhibits mouse type C viral reverse transcriptase was used to ensure that the enzyme activity observed was viral in origin (1). Virus transmission was measured by the XC plaque assay (15). Potential recombinant virus was cloned in microtest II plates (Falcon Plastics) using procedures described in detail previously (20). Virus neutralization assays were performed by the focus-reduction method (2). Neutralizing antiserum against R-MuLV, prepared in rats carrying a transplantable murine sarcoma virus (R-MuLV) tumor, was supplied by R. Wilsnack, Huntingdon Laboratories. Sera from nonimmunized NZB/BLN mice with natural antibody to endogenous xenotropic mouse type C viruses have been described (2).

Radioimmunoassays for viral polypeptides. Specific immunoassays for the 30,000-mol wt p30 and 12,000-mol wt p12, R-MuLV type C viral polypeptides were performed as previously reported (21, 25). These assays measure the relative abilities of unlabeled viral polypeptides to compete with ^{125}I-labeled R-MuLV p30 or p12 for binding limiting antibody prepared against ether-disrupted R-MuLV.

RESULTS

Physiological classification of representative R-MuLV ts mutants. Each of the six ts mutants of R-MuLV examined in the present study was previously shown to be defective in a replication step subsequent to absorption and penetration (17, 18). Some of these have been shown to be ts in steps prior to synthesis of the major virion structural polypeptide, p30 (17). Recently, highly sensitive and specific immunological methods have become available for quantitation of a second type C virion polypeptide, designated p12 (21, 25). Thus, tests were

performed to compare the production of p12 and p30 in cells infected by various mutants at the permissive (31 C) and nonpermissive (38 C) temperatures. As shown in Table 1, three mutants were ts for synthesis of p12, as well as p30. In contrast, the three other mutants tested synthesized both polypeptides at similarly high levels at the two temperatures. The relative as well as absolute quantities of p12 and p30 viral antigens detected in cells infected with each of these latter mutants and wild-type R-MuLV were indistinguishable.

The above results, in addition to previously reported properties of the R-MuLV ts mutants used in the present study, are summarized in Table 2. Of several distinct physiological classes, three mutants, ts 17, 19, and 29, comprise a class which is ts in replication steps following new infection prior to synthesis of p30 and p12 (Table 1). Each of these three mutants is also defective in helper functions which MuLV provides for establishment of transformation by murine sarcoma virus (18). Cells infected with representative class 2 mutants, ts 25 and ts 26, express equal levels of p30 and p12 at both permissive and nonpermissive temperatures; only at 31 C are detectable amounts of progeny virus released. Finally, with class 3 mutants, represented by ts 28, infected cells release type C virus at comparable levels at both 31 and 38 C. However, the virus released at 38 C is noninfectious, even when assayed at the permissive temperature (17).

TABLE 1. *Measurement of production of two type C viral polypeptides at 31 and 39 C after infection with wild-type or ts R-MuLV*[a]

Cells infected with:	Increase in levels of cell-associated viral polypeptides[b]					
	31 C			38 C		
	p30	p12	p30/p12	p30	p12	p30/p12
Wild-type R-MuLV	520	185	2.8	540	160	3.4
ts 17	400	130	3.1	<2	<1	
ts 19	410	100	4.1	<2	<1	
ts 25	190	60	3.2	220	70	3.1
ts 26	510	150	3.4	480	190	2.5
ts 28	210	75	2.8	190	80	2.4
ts 29	610	190	3.2	<2	<1	

[a] Exponentially growing NIH/3T3 cells, pretreated with polybrene (2 μg/ml) for 24 h, were infected with wild-type and ts R-MuLV at the permissive and nonpermissive temperatures at a multiplicity of infection of around 1. After a single cycle of virus replication, 24 h at 38 C or 48 h at 31 C, the level of cell-associated p30 and p12 was determined by radioimmunoassays (21, 25). As previously reported, uninfected NIH/3T3 cells contained approximately 20 ng of p30 and 7 ng of p12 per mg of cell protein (22). These values were subtracted from the values obtained for the infected cells. The results represent the mean of three separate experiments.

[b] Expressed as nanograms of viral polypeptide per milligram of cell protein.

TABLE 2. *Summary of properties of representative R-MuLV ts mutants*

Virus clone[a]	Virus replication step completed at nonpermissive temperature:				
	Absorption and penetration	Viral antigen synthesis		Virion synthesis	Infectious virus synthesis
		p30	p12		
Wild-type R-MuLV	+	+	+	+	+
ts Mutant					
Class 1					
ts 17	+	−	−	−	−
ts 19	+	−	−	−	−
ts 29	+	−	−	−	−
Class 2					
ts 25	+	+	+	−	−
ts 26	+	+	+	−	−
Class 3					
ts 28	+	+	+	+	−

[a] Physiological characterization of these six representative R-MuLV ts mutants is based on data previously published (17, 18, 20), and the results summarized in Table 1.

Preliminary attempts to detect genetic recombination. In preliminary attempts to detect genetic recombination, NIH/3T3 cultures were infected with ts mutants, singly or in pairs, at 31 C. After 7 days, tissue culture fluids were harvested and assayed for XC plaque formation at 38 C. Whereas the supernatant fluids from cultures simultaneously infected with pairs of mutants appeared to have higher titers of XC plaque-forming activity than those of singly infected cultures, the XC plaques obtained were small and difficult to score. Further, XC plaque formation was nonlinear with virus dilution, suggestive of an interference pattern. This might result from competition for cell receptors between ts mutant virus in large excess of any wild-type recombinant virus. Further, if ts mutant virus, defective in late replication steps, infected sufficient numbers of cells at the high temperature, it might also interfere with spread

of any recombinant virus and thereby inhibit formation of XC plaques.

Evidence for genetic recombination between R-MuLV ts mutants. In an attempt to overcome the above problems in detecting and quantitating recombination between pairs of ts mutants, an intermediate step in the assay was introduced to eliminate as much as possible interfering ts virus. At 7 days after infection with mutant pairs at 31 C, culture fluids were used to infect NIH/3T3 cells at 38 C. Supernatant fluids were harvested after 5 days of incubation at 38 C and assayed for XC plaque-forming activity at 38 C. It was reasoned that a short period of growth at 38 C would preferentially select for growth of recombinant virus. Using this procedure the XC plaques that formed were distinct, and the titration was linear.

By this method of assay, evidence for genetic recombination was obtained with each of the mutant pairs tested. As shown in Table 3, cultures infected with individual mutants gave rise to no more than three or four plaques when assayed for XC plaque activity at 38 C. In contrast, simultaneous infection with ts mutant pairs resulted in titers ranging from 30 to 270 PFU/ml. A representative assay showing the XC plaque-forming activities of virus obtained after infection of NIH/3T3 cells with ts 17, ts 19, or with both is shown in Fig. 1.

Replication of recombinant R-MuLV clones at 31 and 38 C. To determine whether the potential recombinants detected above were genetically stable, representative virus clones were isolated. NIH/3T3 fibroblasts were infected at the nonpermissive temperature (39 C) at 0.5 $\log_{10}$ dilutions with potential recombinants ts 17-25, ts 17-29, and ts 25-29. After 24 h cells were trypsinized and transferred to microtest II plates at 100 cells per well. After 20 days of incubation at 38 C, 10^3 XC cells were added to each microtest well, and 4 days later virus-positive wells were identified by the presence of large multinucleated cells as described previously (20). Several virus clones were selected from microtest plates with no more than 10% positive wells and were grown to high titer for further examination. Each virus clone was tested by the polymerase induction assay for transmission to NIH/3T3 at 31 and 38 C. The results (Table 4) show that wild-type R-MuLV transmitted to NIH/3T3 with equal efficiency at both temperatures, whereas each of the original ts mutant clones was detectably transmissible only at 31 C. The potential recombinant clones tested all transmitted equally well at both temperatures and were, in this way, similar to wild-type R-MuLV. These findings provide evidence for the formation of stable recombinants. An alternative possibility, that a heterozygote initially formed between two complementing parental ts mutants continues to grow as a heterozygote in a very stable manner such as to be indistinguishable from the wild-type R-MuLV, is considered unlikely.

TABLE 3. *XC plaque-forming activity of culture fluids of pairs of mutants after coinfection at 31 C and amplication at 38 C*

ts Virus clone	XC plaque titer at 38 C (PFU/ml)					
	ts 17	ts 19	ts 25	ts 26	ts 28	ts 29
ts 17	3					
ts 19	180	0				
ts 25	100	210	1			
ts 26	150	190	200	0		
ts 28	90	130	80	175	3	
ts 29	30	250	130	80	270	4

[a] Exponentially growing NIH/3T3 cultures, pretreated with polybrene (2 μg/ml) for 24 h, were infected at 31 C with individual ts mutants at a multiplicity of infection of 2 or with pairs of mutants, each at a multiplicity of infection of 1. After 7 days of incubation at the permissive temperature, tissue culture fluids were harvested and used to infect cultures of NIH/3T3 at 38 C. Culture fluids were harvested 5 days later and titrated at the nonpermissive temperature (38 C) for XC plaque-forming activity on NIH/3T3 (15). The results represent mean values from three separate experiments.

Comparison of host range and serological properties of recombinant R-MuLV clones and endogenous type C virus of NIH Swiss mouse cells. Recent evidence has indicated that stable genetic recombination can occur between exogenous and endogenous type C viral genetic information (19, 29). Therefore, the possibility was considered that the recombinants detected in the present studies might have resulted from genetic interaction between exogenous ts mutant and endogenous type C viral genetic information of NIH Swiss cells. The host ranges and serological properties of several recombinant clones and a recent isolate of an endogenous type C virus of NIH Swiss cells were compared (Table 5). The endogenous NIH Swiss virus was highly infectious for cells of a human tumor line, A673, but did not detectably replicate in NIH Swiss mouse cells. In contrast, R-MuLV and the recombinant clones grew efficiently in NIH/3T3 but were noninfectious for A673 cells. Further, antiserum to R-MuLV at a dilution that completely neutralized R-MuLV but less than 20% of NIH virus infectivity inhibited more than 95% of

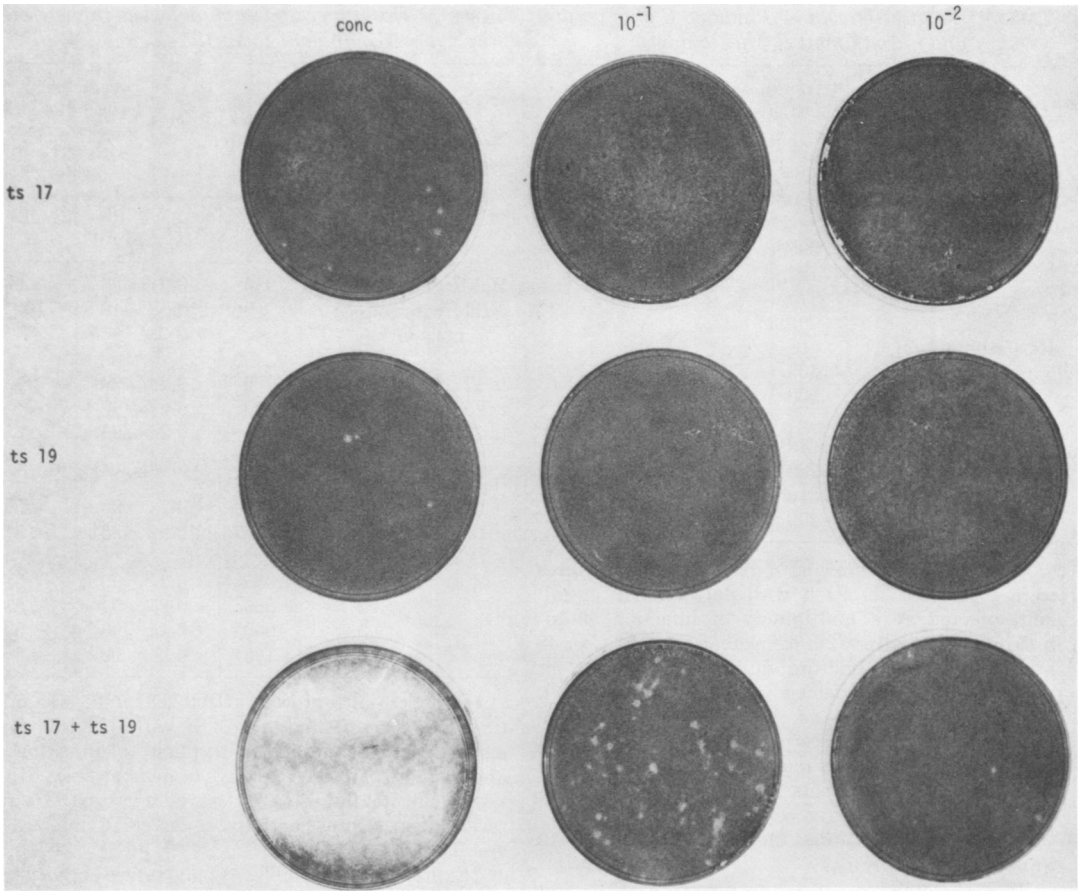

FIG. 1. *XC plaque-forming activity of culture fluids of ts 17 and ts 19 after coinfection at 31 C and amplification at 38 C. Exponentially growing NIH/3T3 cultures, pretreated with polybrene (2 μg/ml) for 24 h, were infected at 31 C with ts 17 and ts 19 singly, or together, at a multiplicity of infection of 2. After 7 days of incubation at 31 C, tissue culture fluids were harvested and used to infect NIH/3T3 cultures at 38 C. Culture fluids were harvested 5 days later and assayed directly and at 10^{-1} and 10^{-2} dilutions for XC plaque-forming activity on NIH/3T3 (15).*

the infectivity of each recombinant clone. In contrast, normal sera of NZB mice that markedly neutralized NIH Swiss virus (2) contained no significant activity against R-MuLV or any of the recombinant clones (Table 5). The above findings indicate that the host range and serological properties of the recombinant viruses tested were indistinguishable from those of the parental wild-type R-MuLV.

DISCUSSION

Conditional lethal mutants have proven extremely useful in the genetic analysis of virus replication in bacterial phage systems and with several groups of animal viruses (7). In the present studies, the feasibility of utilizing MuLV ts mutants in the genetic analysis of the type C viral genome has been examined. The evidence indicates virus recombination can occur between functionally distinct classes of ts mutants. These results are consistent with those of studies in which sarcoma virus-transforming information and host range markers of avian leukosis viruses have been shown to genetically recombine (12, 26, 27), and with a recent report of genetic interaction between two Moloney MuLV ts mutants (30). Stable genetic recombination between exogenous avian or mouse type C viruses with endogenous type C viruses present in cells of the homologous species has also been demonstrated (19, 29). The availability of ts mutants of two different strains of MuLV, Kirsten MuLV (20) and R-MuLV (17), as well as assays that detect differences in their host ranges and immunological characteristics (2, 21), should make it possible to define linkages

TABLE 4. *Relative growth at 31 and 38 C of ts mutant and recombinant R-MuLV clones*[a]

Virus clone	Transmission of infectious virus at:		(31 C/38 C)
	31 C	38 C	
Wild-type R-MuLV	360	340	1.1
ts 17	305	0.4	760
ts 25	190	0.2	950
ts 29	375	0.5	750
Recombinant of:			
ts 17 + ts 25:	305	290	1.1
ts 17 + ts 29:	370	410	0.9
ts 25 + ts 29:			
1	210	250	0.8
2	280	220	1.3
3	310	350	0.9
4	270	285	1.0

[a] Exponentially growing NIH/3T3 cells cultures were pretreated for 24 h with polybrene (2 μg/ml) and infected at a multiplicity of infection of approximately 1. After 72 h of incubation at 31 C or 48 h at 38 C, tissue culture fluids were assayed for poly(rA)-oligo (dT)-directed poly(dT) synthesis (17). Results are expressed as picomoles of polymerase activity ([^{3}H]TMP) incorporated per milliliter of tissue culture fluid and represent mean values from three separate experiments.

TABLE 5. *Host range and immunological properties of recombinant R-MuLV isolates*[a]

Kirsten murine sarcoma virus pseudotype	Transmission to[b]:		Reduction in focus formation[c] after treatment with antisera against:	
	NIH/3T3	A673	R-MuLV	NZB-MuLV
Wild-type R-MuLV	980	<0.2	98	<5
NIH Swiss endogenous virus	<0.2	210	18	100
ts 17	750	<0.2	99	<5
ts 25	1,200	<0.2	95	<5
ts 29	875	<0.2	93	<5
Recombinant of:				
ts 17 + ts 25:	950	<0.2	98	<5
ts 17 + ts 29:	910	<0.2	98	<5
ts 25 + ts 29:				
1	1,050	<0.2	95	<5
2	880	<0.2	97	<5
3	900	<0.2	93	<5
4	1,125	<0.2	96	<5

[a] Exponentially growing NIH/3T3 and A673 cultures were pretreated for 24 h with polybrene (2 μg/ml) and infected at a multiplicity of infection of approximately 1. After 10 days of incubation at 31 C, tissue culture fluids were assayed for type C viral reverse transcriptase activity as described in footnote *a* of Table 4.

[b] Expressed as picomoles of polymerase activity per milliliter of culture fluid.

[c] Neutralization tests were performed by the focus-reduction method as described previously (2).

between ts mutations and these markers in genetic recombinants.

The mechanism of recombination is as yet unknown. If the type C virus possessed a segmented genome, the segments might be expected to undergo random reassortment in a manner similar to that reported for RNA viruses such as influenza (10, 14) and reovirus (8, 16). Analysis of the genetic complexity of type C viral RNA by DNA reassociation kinetics has suggested a mol wt of around 10^7 (23). Since the virus contains a single-stranded 70*S* RNA which readily disassociates into 35*S* subunits (3, 5) with a mol wt of around 2×10^6 to 3×10^6, these latter units could represent viral RNA segments. If, however, the genetic size of type C virus is only around 2×10^6 to 3×10^6 daltons (P. H. Duesberg, P. K. Vogt, K. Beeman, and M. Lai, Cold Spring Harbor Symp. Quant. Biol., in press), corresponding to the molecular weight of the 35*S* subunit (6), subunit reassortment would appear to be much less likely as a mechanism for genetic recombination (28).

Evidence from other studies supports the possibility that certain RNA viruses can undergo classic genetic recombination (4, 10, 28). Type C viruses appear to possess certain properties unique among known RNA viruses. Accumulating evidence indicates that these viruses replicate through a DNA intermediate which at some stage in infection becomes stably integrated into the host cell genome. Thus, recombination might occur by mechanisms involving the DNA intermediates if not the viral RNA genome itself. In this regard, there is a need for two cycles of infection so that the formation of distinct XC plaques at the nonpermissive temperature may reflect a specific requirement for recombination rather than simply be a means of eliminating interfering ts mutant virus. In fact, independent evidence from studies with avian sarcoma virus ts mutants supports this possibility (Wyke et al., manuscript in preparation).

Although the present results do not discriminate between the alternative possibilities of subunit reassortment and classic genetic recombination, the fact that several ts mutant pairs

showed evidence of recombination favors the latter model. It is unlikely that the type C viral genome would consist of a sufficient number of segments that each of several ts defects studied would involve a different one. Extension of these studies to an examination of the recombination frequencies between these and ts mutants of both R-MuLV and Kirsten MuLV should help to define the genetic structure of mammalian type C RNA viruses.

ACKNOWLEDGMENT

We thank Janet A. Steel and Pamela M. Johnson for their excellent technical assistance.

This work was supported by Public Health Service contract NCI-73-3212 from the Special Virus Cancer Program of the National Cancer Institute.

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