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Transformation-Defective Mutants of Rous Sarcoma Virus with *src* Gene Deletions of Varying Length

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The RNAs of transformation-defective (*td*) deletion mutants of the Schmidt-Ruppin strain of Rous sarcoma virus were found to vary in size when compared by polyacrylamide gel electrophoresis. Three of seven *td* mutants appeared to recombine with a mutant of Rous sarcoma virus (Schmidt-Ruppin), which has a temperature-sensitive sarcoma (*src*) gene and is termed *ts*68, to give rise to recombinants with a reduced temperature sensitivity. The results suggested that different clones of *td* mutants exist: some in which the *src* gene appears to be deleted, and others in which the *src* gene is only partially deleted. A direct correlation between RNA size and the extent of *src* gene deletion measured by recombination was not obtained, possibly because the recombination assay could only detect *src* sequences homologous to the lesion(s) of *ts*68, whereas the electrophoretic analysis of the RNA measured *src* deletions as well as other possible alterations of the RNA.

Transformation-defective (*td*) mutants of Rous sarcoma virus (RSV) obtained by spontaneous mutation of nondefective (*nd*) RSV are viruses that retain all replicative functions but have lost the ability to induce transformation in infected cultures (11, 15, 19). It has been demonstrated that the genome RNA of typical, nonconditional *td* mutants is of rather uniform size, smaller than that of the transforming parental virus, and, in general, very similar to that of naturally existing avian leukosis viruses such as Rous-associated virus 2 (RAV-2) (2, 4, 5, 15). Such nonconditional *td* viruses were shown to lack genetic elements capable of complementing or recombining with the sarcoma (*src*) gene of *nd* sarcoma viruses (2). Their RNA is thought to reflect the minimal complexity of an independently replicating avian oncovirus. The difference between the RNA sizes of *nd* and *td* viruses was utilized to identify viral RNA sequences responsible for sarcoma virus-specific transformation (9, 14, 20, 21) and also for preparation of complementary DNA specific for the *src* gene (7, 17). The mechanism responsible for forming this type of deletion mutant is not known, but it has been suggested that the deletion of *src* occurs at some time during the synthesis or integration of proviral DNA (8, 16, 20, 21).

In this communication, we report evidence suggesting that some isolates of *td* mutants spontaneously derived from the Schmidt-Ruppin strain of RSV, subgroup A (SR-RSV-A), have genome RNA in which the *src* gene is only partially deleted and which retain genetic elements capable of recombining with the *src* gene of *nd* virus.

td mutants were isolated from cultures infected with high dilutions of SR-RSV-A, as described (11, 15, 19), and were purified by one cycle of end-point dilutions. Seven independent isolates used in this study were extensively examined for absence of transforming virus. First, chicken embryo fibroblasts (gs^-chf^-, C/E type obtained from SPAFAS, Inc., Norwich, Conn.) were exposed to these *td* viruses and subcultured at 3- to 4-day intervals a total of 10 times. No transformed cells were observed. At each subculture, the culture fluids were tested for focus-forming virus by inoculating various dilutions into fresh chicken embryo cells. No foci were demonstrated. These observations showed that the *td* mutants were free of transforming virus. That no transforming virus was recovered after many passages suggests that the mutants contain deletions within the *src* gene.

These *td* mutants were inoculated into clones of cells transformed by two mutants of SR-RSV-A. One mutant, termed *ts*68/env^-, is a recombinant between *tsNY*68SRA and *rdNY*8SR (known as *ts*68 and N8, respectively) (13), which is temperature-sensitive (*ts*) in transformation as is *ts*68 (10) and deficient in envelope glycoprotein gene (*env*) as is N8 (3, 12). Another

mutant, *ts*68α/*env*⁻, is similar to *ts*68/*env*⁻ but also has a defective *pol* gene (13). The genotypes of recombinants *ts*68/*env*⁻ and *ts*68α/*env*⁻ can be expressed as src^{ts} env⁻ *pol*⁺ and src^{ts} *env*⁻ *pol*⁻, respectively, according to the expressions used in a previous paper (13). Because of their defectiveness, these viruses can only infect cells as pseudotypes, or mixed phenotypes, usually generated by simultaneous infection with leukosis virus. Cells singly infected with such pseudotypes fail to produce infectious virus and are termed nonproducer cells. Nonproducer colonies of transformed chicken cells singly infected with RAV-1 pseudotypes of *ts*68/*env*⁻ or *ts*68α/*env*⁻ were isolated from soft-agar colonies. Because of the absence of viral envelope glycoprotein synthesis, these colonies of chicken cells produce only noninfectious particles lacking envelope glycoprotein spikes and, thus, remain susceptible to any subgroup of avian oncoviruses. Infection of these colonies with standard avian leukosis virus such as RAV-1, RAV-2, and RAV-7 produced pseudotypes of *ts*68, and the recovered transforming virus was temperature sensitive for transformation to the same extent as was the original *ts*68 subgroup A (Table 1).

Infection with four *td* mutants (*td*101, -103, -105, -106) produced pseudotypes of *ts*68/*env*⁻ with essentially the same temperature sensitivity as those produced by RAVs, as measured by the ratio of focus formation at 41 and 37°C. However, RSV progeny obtained after superinfection with three other *td* mutants (*td*107, -108, and -109) formed foci at 41°C at approximately 10 to 100 times higher efficiency. As shown in Table 1, this observation was reproducible with different colonies of cells infected with either *ts*68/*env*⁻ or *ts*68α/*env*⁻. The RSV progeny obtained by superinfection with these three *td* mutants also produced soft-agar colonies at 41°C with an efficiency comparable to that of focus formation.

Forty-three colonies produced at 41°C by progeny of the cross *ts*68/*env*⁻ × *td*107 were isolated. All of the virus clones recovered from each colony have essentially the same plating efficiencies at 37 and 41°C (data not shown). Therefore, transformation at 41°C by these viral products was apparently due to formation of stable recombinants similar to the wild type. However, closer observation of the properties of transformed cells induced by these recombinants showed that they were not exactly like wild-type RSV-transformed cells at 41°C. These cells had a tendency to become flat, so that recognition of transformed morphology was occasionally not easy, although they retained polygonal shapes and were, thus, distinguishable from cells infected with *ts*68-A at 41°C. Partial temperature sensitivity at 41°C was shared by all 43 colonies described above. Other phenotypes of chicken cells transformed by the recombinants with partial temperature sensitivity have not been studied. However, since these viruses produced foci at 41°C, they seem to differ from some partially *ts* mutants of RSV reported to produce transformed cells temperature sensitive for focus formation and many other properties, but not temperature sensitive for colony formation or plasminogen activator (1).

Since the above-described results indicated differences among *td* mutants that were possibly related to the extent of the deletion in these mutants, we examined the size of viral RNA by electrophoresis in polyacrylamide gels (4). In

TABLE 1. *Infectivity of viruses recovered from cells transformed with ts68/env⁻ or ts68α/env⁻ and superinfected with various td mutants of SR-RSV-A*[a]

Superinfecting virus	Ratio of no. of foci at 41 and 37°C				
	1[b]	2	3	4	5
RAV-1	0.0009			0.00092	0.0009
RAV-2	0.0005				0.0004
RAV-7	0.0004		0.0002		0.0006
*td*101	0.00067	0.00034	0.00025		0.0005
*td*103			0.00022		
*td*105	0.0013	0.0022	0.00050	0.0015	0.0004
*td*106		<0.0030	0.0030	0.0017	
*td*107	0.060	0.083	0.072	0.062	0.060
*td*108	0.016	0.020		0.013	
*td*109		0.104		0.031	0.013

[a] Clones of chicken cells transformed by *ts*68/*env*⁻(clones 1 through 4) or by *ts*68α/*env*⁻(clone 5) were superinfected with various RAVs and *td* mutants of SR-RSV-A at a multiplicity of infection of about 0.1. Focus-forming titers of viruses in culture fluids harvested at 7 days after infection were assayed on chicken cells at 37 and 41°C. The ratios of focus formation of SR-RSV-A and *ts*68-A at these two temperatures are on the average 1.1 and 0.00046, respectively. The ratios considered significantly higher than typical *ts* mutants are underlined.

[b] Clones of transformed cells.

estimating the size of viral RNA from their electrophoretic patterns, the following is considered: all preparations of viral RNA consist of intact and degraded species in various ratios (4, 5). Consequently, the position of the peak of a given RNA population is influenced by the ratio of intact to faster-migrating, degraded RNA species. We have, therefore, used the trailing edges of RNA peaks, which are defined by intact RNA species only, as a measure for size comparison. In addition it is noted that the mobility of an RNA is not only determined by its size but also by its conformation, and our method cannot directly distinguish between these two factors, thus measuring only an apparent size. As shown in Fig. 1, the *td* mutant RNAs were definitely smaller than the RNA of Prague strain of RSV-B (abbreviated as PR-B, which is known to contain RNA of the same size as that of SR-RSV-A), but were similar in size to that of *td* PR-RSV-B (*td*PR-B), which was used as a standard for *td* viral RNA. Some *td*SR-A RNAs had the same apparent size as *td*PR-B RNA (*td*101, *td*105). Some were slightly smaller (*td*106, *td*109), and some were slightly larger (*td*103, *td*107, *td*108) than the *td*PR-B RNA standard. In repeated runs, a shoulder was observed at the slower-migrating portion of the RNA peak of *td*107. This suggested that the stock of *td*107 consisted of two viruses, a major component containing smaller-sized RNA, which comigrated with *td*PR-B, and a minor component containing larger-sized RNA. The difference in size estimates of RNAs of this larger component of *td*107 and of *td*PR-B was equivalent to 35 to 45% of the size of the *src* gene deletion in *td*PR-B (cf. Fig. 1A and F). These analyses clearly demonstrated variation in the electrophoretic mobilities and, consequently, in sizes and structures of genome RNAs of different isolates of *td* mutants of SR-RSV. We conclude that complete deletions, as well as partial deletions of the *src* gene (and possibly other alterations of the RNA), were observed.

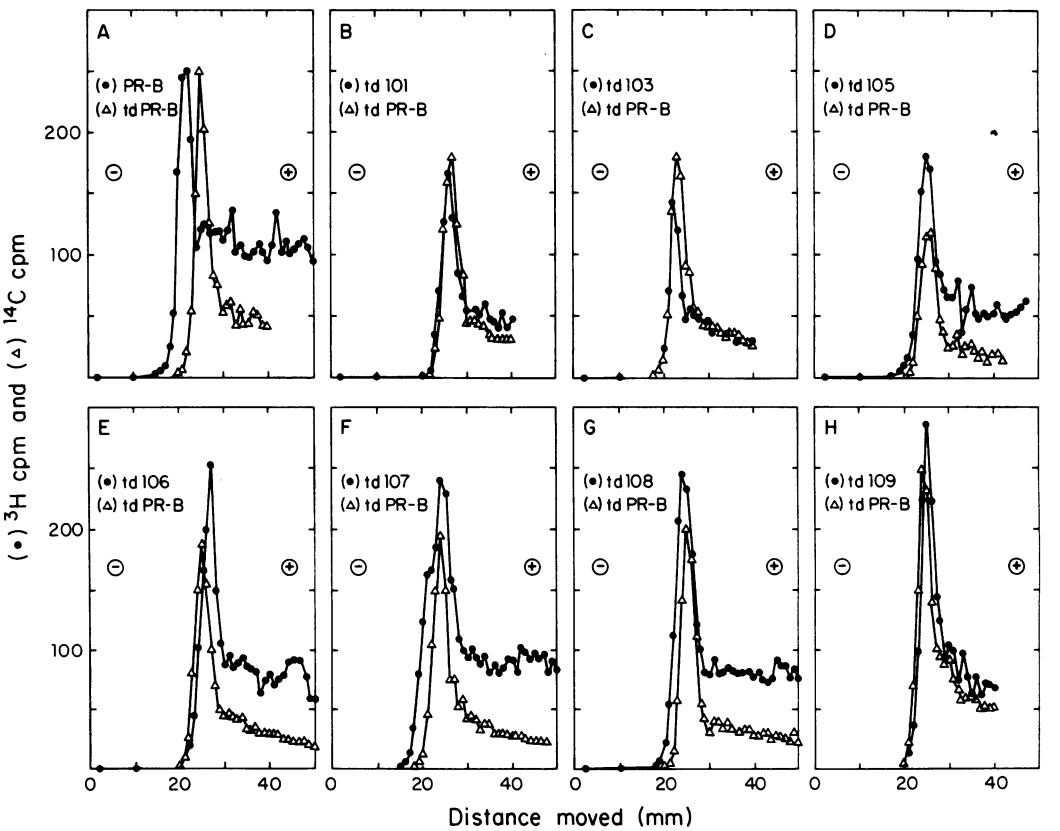

FIG. 1. *Analysis of td mutant RNA size. Appropriate amounts of radioactively labeled 60 to 70S RNA, extracted from virus harvested at 3- to 5-h intervals from infected cells, were mixed, heated in electrophoresis sample buffer, and subjected to electrophoresis in 2% polyacrylamide gels as described before (4). The RNA of PR-RSV-B and tdPR-RSV-B were used as markers for a and b classes of viral RNA (5).*

A correlation between apparent size of the RNA and capacity to form recombinants with partial temperature sensitivity was seen with *td*107 and *td*108 but not with *td*109 and *td*103. *td*103 contained RNA larger than *td*PR-B but produced pseudotypes of *ts*68/*env*$^-$, which are indistinguishable from original *ts*68 in temperature sensitivity. *td*109 produced pseudotypes with increased plating efficiency at 41°C but contained small RNA. This could be explained as follows: *td*103 may contain residual *src* RNA but may not include the sequences corresponding to temperature-sensitive lesions in *ts*68 and would, consequently, be unable to recombine in this function. *td*109 may contain residual *src* sequences homologous to the *ts* lesions of *ts*68 that were either too small to be detected electrophoretically or were accompanied by small deletions or conformational changes in other segments of the RNA that obscured the contribution of the remaining *src* sequences.

Since *td*107 was apparently a mixture of two virus components with different sizes of RNA, the virus was further purified by another cycle of end-point dilution. The virus isolated from the terminal dilution (10^{-6}), termed *td*107A, was found to contain RNA indistinguishable in size from that of *td*PR-B (data not shown). The progeny of the cross between *ts*68/*env*$^-$ and *td*107A had a low plating efficiency at 41°C similar to the original *ts*68. Therefore, at least in this case, the component in *td*107 with larger genomic RNA appeared to be responsible for formation of recombinants with partial temperature sensitivity.

The formation of recombinants with partially restored temperature stability indicates that genetic interaction had taken place within the *src* gene, thus suggesting that portions of the *src* gene were still present in the RNA of some *td* mutants. However, the properties of newly formed recombinants were not entirely the same as the wild-type SR-RSV. This partial restoration may be explained if one assumes that *ts*68 has mutations at more than one locus within the *src* gene. Some, but not all, of these mutations would be retained in the recombinants formed with *td* mutants, and, as a consequence, the reversion or leaky expression of transformation of the recombinants would occur with a higher frequency than it does with *ts*68. That all colonies made by the recombinants expressed partial *ts* properties seems to favor leaky expression at 41°C of a partially restored *src* gene rather than an enhanced spontaneous reversion to wild type. Further studies on the exact location of the deletions of each *td* mutant would be necessary to evaluate the above explanation. These studies would be useful for understanding the expression of *src* gene products and for localizing the lesions of *ts* mutants of RSV.

A variation in the length of the RNA of *td* mutants has been reported by Stone et al. (18) with a *td* derivative of SR-RSV-D treated with hydroxylamine (6). Recently, spontaneous *td* mutants of RSV with limited deletions have also been isolated by M. C. Lai, S. Hu, and P. K. Vogt (personal communication) and M. Yoshida and Y. Ikawa (personal communication).

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