Transduction of Proto-*src* Sequences in Tissue Culture by a Molecular Clone of Transformation-Defective Rous Sarcoma Virus with an Internal *src* Deletion

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The sporadic appearance of nondefective (*nd*) Rous sarcoma virus (RSV) from cells in tissue culture infected with a molecular clone of transformation-defective RSV was examined. Southern analysis of extrachromosomal, virus-specific DNA of three independent *nd*RSV isolates in each case indicated restoration of an isogenic *src* by homologous recombination with cellular proto-*src*. The frequency of transduction was estimated by fluctuation analysis to vary between one transduction per 0.4×10^7 to 1.6×10^7 infected cells.

In this report, homologous recombination between retroviruses and cellular sequences is examined with a molecular clone of a transformation-defective (*td*) mutant of the avian Rous sarcoma virus (RSV) in which the oncogene, *src*, contains an internal deletion of 1.1 kilobases (kb). Recombination through the Δ *src* sequences remaining in the *td*RSV mutant with homologous cellular sequences present at the single proto-*src* locus in chicken DNA (18, 25, 29) would be expected to generate wild-type nondefective (*nd*) RSV. Such recombination has been thought to account for *nd*RSV in tumors of animals inoculated with naturally occurring *td*RSV mutants (5, 8, 22, 32), although use of these mutants has been criticized (22) because of the possibility of heterogeneous, nonoverlapping *src* mutations in biologically purified stocks of naturally occurring *td*RSV (13, 19, 33) which might generate *nd*RSV by cross-reactivation. However, *nd*RSV has been obtained from tumors induced by a molecular clone of *td*108, a naturally occurring *td*RSV mutant with an internal *src* deletion (33), and in tissue culture from three constructed mutants with various 5' *src* deletions (4). The work presented here confirms the appearance of *nd*RSV from cells in a culture infected with a molecularly cloned *td*RSV and, in addition, examines several independent *nd*RSV isolates biochemically to distinguish between recombinant virus or possible contamination by wild-type RSV. The recombination was measured by fluctuation analysis of the appearance of infectious *nd*RSV, which tests the assumption that transduction occurs randomly in time, in a series of experiments initiated from very small inocula of endpoint-diluted *td*RSV.

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The *src* deletion mutant was prepared from recombinant plasmid-Prague A (PrA) RSV DNA (pJD100) (9) by deletion of sequences spanning two *Hinc*II cleavage sites within the *src* gene; approximately 0.4 kb of 5' *src* sequences and 0.2 kb of 3' sequences remained in the mutant, here termed *ptd*Berk101. This plasmid and pJD100 were obtained from J. Thomas Parsons. *ptd*Berk105, a derivative of *ptd*Berk101 containing an unambiguous biochemical marker in viral sequences, was prepared by *Not*I linker insertion in the *Pvu*I site of the 3' long terminal repeat (LTR). Viruses were

recovered from cloned DNA by transfection (7) into C/E, *chf*-negative chicken embryo fibroblast (CEF) cultures (SPAFAS, Inc., Norwich, Conn.). During subsequent passage of cells treated with either *ptd*Berk101 or *ptd*Berk105, discrete transformed foci developed in some tertiary cultures maintained in monolayer for 2 weeks; the number of cultures with foci and the number of foci per culture varied with the transfection. From many cultures tested, *nd*RSV was isolated by focus formation (30), suggesting that recombination had occurred, either with *td*RSV recovered from transfected DNA or directly with the transfected DNA. Homologous recombination in certain cases is known to occur between transfected DNA and cellular sequences (1, 6, 12, 15, 26). However, in this case it is unlikely that recombination occurred at this stage and much more likely that it occurred during subsequent infection by virus, since (i) foci appeared only late during passage of the cultures (also observed previously [4], with the exception of NY18-3); (ii) following transfection, after treated cultures were split into separate subcultures, the ratio of subcultures with foci to total subcultures varied with the transfection; and (iii) immunocytochemical analysis with α -p19*gag* antibody (28) of cultures overlaid with agar posttransfection indicated a low efficiency of transfection, with <700 infectious centers recovered from 10 μ g of purified plasmid DNA per 5×10^6 CEF (data not shown).

To test the assumption that recombination did occur with infecting virus, *td*RSV stocks were first purified by endpoint

TABLE 1. Absence of *nd*RSV in primary endpoint-diluted *td*RSV stocks^a

Virus	Inoculum (μ l)	No. of infected cultures with <i>nd</i> RSV foci/total infected cultures
<i>td</i> Berk101	1.0	0/16
	10	0/24
	100	0/71
<i>td</i> Berk105	0.1	0/20
	1.0	0/15
	10	0/13
	100	0/16

^a Primary endpoint-diluted *td*Berk101 and *td*Berk105 stocks were tested for the presence of *nd*RSV by inoculation, as indicated, of 5×10^5 CEF per 35-mm dish. Cultures were maintained for 2 weeks for development of foci.

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FIG. 1. Southern hybridization analysis of extrachromosomal, virus-specific DNA. The viruses analyzed and the endonucleases used were as follows: PrA RSV (recovered from pJD100), undigested (lanes 1) or digested with *Bgl*II (lanes 2), *Bgl*II and *Not*I (lanes 3), *Pst*I (lanes 4 and 34), *Mst*II (lanes 32 and 37), *Mst*II and *Bgl*II (lane 33), and *Pvu*II (lane 38); *tdBerk*101 digested with *Mst*II (lane 35); *tdBerk*101-135 (from blood of a tumor-negative bird injected with *tdBerk*101) digested with *Mst*II (lane 36); *ndBerk*105-1.1, undigested (lanes 5) or digested with *Hpa*I (lanes 6), *Bgl*II (lanes 7), *Bgl*II and *Not*I (lanes 8), and *Pst*I (lanes 9); *tdBerk*105, undigested (lane 10) or digested with *Hpa*I (lanes 11), *Bgl*II (lanes 12), and *Bgl*II and *Not*I (lanes 13); *ndBerk*105-3.1, undigested (lanes 14) or digested with *Bgl*II (lanes 15), *Bgl*II and *Not*I (lanes 16), and *Pst*I (lanes 17); *ndBerk*105-4.1, undigested (lanes 18) or digested with *Hpa*I (lanes 19), *Bgl*II (lanes 20), *Bgl*II and *Not*I (lanes 21), and *Pst*I (lanes 22); *ndBerk*101-2 digested with *Mst*II (lane 23), *Mst*II and *Bgl*II (lane 24), *Pst*I (lane 25), and *Pvu*II (lane 39); *ndBerk*101-10.5 digested with *Mst*II (lane 26), *Mst*II and *Bgl*II (lane 27), *Pst*I (lane 28), and *Pvu*II (lane 40); and *ndBerk*101-13.1 digested with *Mst*II (lane 29), *Mst*II and *Bgl*II (lane 30), *Pst*I (lane 31), and *Pvu*II (lane 41). The probe was a purified *gag-pol-env* fragment labeled with 32 P (A) or a purified *src* fragment (B and C). Panels A and B are from the

dilution, and the resulting stocks which contained no detectable *ndRSV* were used for further experiments; no focus-forming virus was seen in the endpoint-diluted *tdBerk*101 or *tdBerk*105 stocks used, in a total of 5×10^7 or 2×10^6 IU tested, respectively (Table 1). The endpoint-diluted *tdBerk*105 stock was examined by Southern analysis (27) of purified extrachromosomal viral DNA (10, 20) extracted from infected quail QT6 cells. Autoradiographs of Southern blots are shown in Fig. 1, in which viral DNAs have been differentially hybridized with viral or *src* probes; the expected sizes of fragments generated by restriction endonuclease digestion, with sequence homology either to the purified viral *gag-pol-env* probe (6.9 kb, *Sst*I resistant) or to the *src* probe (1.5 kb, *Mst*II resistant), are given in Table 2. The predicted fragments were derived from physical maps of *tdBerk*105, PrA RSV, and putative recombinants between *tdBerk*105 and chicken proto-*src* (Fig. 2); the *gag-pol-env* probe spans the region from the 5'-most *Pst*I site through the entire *gag* and *pol* genes, terminating just past the 3' *Pst*I site in the *env* gene; the *src* probe is the fragment between the internal *Mst*II sites, as indicated in the PrA RSV map (Fig. 2). Undigested DNA visualized with the nick-translated (21) *gag-pol-env* probe showed predominantly a single band corresponding to open circular viral DNA (Fig. 1A, lane 10); presumably most of the viral DNA in these preparations had been nicked (20). Linearization with *Hpa*I (a single cleavage site, Fig. 2) resulted in the predicted single 8.2-kb fragment visualized with either the *gag-pol-env* probe (Fig. 1A, lane 11) or the *src* probe (Fig. 1B, lane 11). Digestion with *Bgl*II yielded the predicted 5.6- and 2.6-kb fragments visualized with the *gag-pol-env* probe (Fig. 1A, lane 12) or only the 5.6-kb fragment visualized with the *src* probe (Fig. 2A, lane 12); further digestion with *Not*I yielded the three predicted fragments of 3.9, 2.6, and 1.8 kb visualized with the *gag-pol-env* probe (Fig. 1A, lane 13) or only the 3.9-kb fragment visualized with the *src* probe (Fig. 1B, lane 13). The difference between the 5.6-kb (*Bgl*II-resistant) or 3.9-kb (*Bgl*II- and *Not*I-resistant) fragment of *tdBerk*105 and the respective 6.7-kb (*Bgl*II) or 4.9-kb (*Bgl*II-*Not*I) fragment of *ndBerk*105 DNA (Fig. 1A and B, lanes 15, 16, 20, and 21), electrophoretically separated in the same agarose gel, corresponds in size to the extent of the Δ *Hinc*II deletion in *tdBerk*105 (Fig. 2). The same difference is also seen between the *Mst*II-resistant *src* fragment of *tdBerk*101 (0.4 kb) and that of PrA RSV (1.5 kb) (Fig. 1C, lanes 35 and 37). The presence of the 0.4-kb *Mst*II-resistant *src* fragment in *tdBerk*101-135 (Fig. 1C, lane 36), a virus recovered from the blood of a tumor-negative chicken inoculated with *tdBerk*101, indicates that the Δ *src* sequences present in the *tdRSV* may be relatively stable during extended passage of the virus; however, the structure was not analyzed in further detail.

To show that *ndRSV* may arise spontaneously from cultures infected with *tdRSV*, fluctuation analysis of recombination in tissue culture was performed in parallel cultures (3×10^5 CEF in 35-mm dishes) inoculated with primary endpoint-diluted *tdRSV* stocks; titers of the viral stocks were determined immunocytochemically with α -p19*gag* antibody (28) to be 7×10^6 IU/ml for *tdBerk*101 and 9×10^5 IU/ml for

same filters, washed and reprobbed. Viral DNAs in panels A and B had been purified by equilibrium density centrifugation in CsCl-ethidium bromide gradients, while those in panel C had not. Restriction endonucleases were from New England BioLabs, Inc., Beverly, Mass., and electrophoresis was done through 0.8% (wt/vol) agarose (SeaKem) gels.

TABLE 2. Predicted fragments generated by restriction endonuclease digestion of circular viral DNA^a

Restriction endonuclease	Length (bp) of fragment in:					
	PrA RSV		<i>ndBerk105</i>		<i>tdBerk105</i> ^b	
	<i>src</i>	<i>gag-pol-env</i>	<i>src</i>	<i>gag-pol-env</i>	<i>src</i>	<i>gag-pol-env</i>
<i>Hpa</i> I	9,312	9,312	9,312	9,312	8,244	8,244
<i>Bgl</i> II	3,500	2,606		2,606		2,606
	3,206	3,500				
		3,206	6,706	6,706	5,638	5,638
<i>Bgl</i> II and <i>Not</i> I		2,606		2,606		2,606
	3,500	3,500				
	3,206	3,206	4,944	4,944	3,876	3,876
				1,762		1,762
<i>Pst</i> I		1,279		1,279		
		908		908		
		3,114		3,114		
		921		921		
		(563)		(563)		
		913		913		
	402		402			
	600		344			
			(256)			
	612		612			
<i>Pvu</i> II		6,208		6,208		6,208
		796		796		796
	1,438	1,438	1,438	1,438	1,240	1,240
	(297)		(297)			
	573		573			
<i>Mst</i> II		7,578		7,578		7,578
	1,485	(249)	1,485	(249)	417	(249)

^a Predicted from the sequence of PrC RSV (23) (and confirmed empirically in pJD100 and *tdBerk101*) and from the proto-*src* sequences (29) or map (25). The fragment size is indicated for predominantly circular viral DNA containing a single LTR. The specific fragments given are those with sequence homology to the *src* or *gag-pol-env* probe (see text). Fragments given in parentheses were not visible in the autoradiograph.

^b Not analyzed with *Pst*I.

FIG. 2. Physical maps of PrA RSV, proto-*src*, and transduced sequences. The linear proviral form of PrA RSV (9) is shown; the region of internal *src* deleted in *tdBerk101* and 105, as well as the site of *Not*I linker insertion, is indicated. Proto-*src* sequences are indicated as exon regions (□) or intron regions (▨) (29) and are not drawn to scale. Positions of restriction enzyme sites were from the same references as in Table 2, footnote a.

FIG. 3. Transformed foci appearing in CEF cultures infected with *tdRSV* during fluctuation tests. Each focus shown is from an independent culture infected with primary endpoint-diluted *tdRSV* stock. Cultures had been infected with *tdBerk101* (a and b) or with *tdBerk105* (c to f). Magnification of unfixed cells in growth medium, $\times 31$.

tdBerk105. Cultures were refed every 3 days with 2-1-1 medium (11) in Ham F-10 containing 0.5% (vol/vol) dimethyl sulfoxide and were transferred into 60-mm dishes and then into 100-mm dishes, at which stage they were maintained for 2 weeks; at this time, foci appeared in some dishes (Fig. 3). Two experiments with *tdBerk101* (Table 3, FT101-1 and FT101-2) and two experiments with *tdBerk105* (FT105-1 and FT105-2) were performed. Media from these cultures, which constitute a parallel series of secondary *tdRSV* stocks, were subsequently tested for the presence of *ndRSV* (5×10^5 CEF per 35-mm dish inoculated with 0.1 ml of medium, maintained for 2 weeks). From 8 to 47% of the secondary stocks were positive (Table 3), indicating that under these conditions most stocks did not contain *ndRSV* and that probably a single transduction had occurred in each positive culture. Reproducibility of tests for *ndRSV* was confirmed by twice testing secondary *tdRSV* stocks, collected 1 week apart (Table 3, FT101-2a and FT101-2b), the only difference being the appearance of an additional positive culture in the second test; sensitivity was determined in reconstruction experiments to be at least 3 focus-forming units. As a control for fluctuation tests, an identical set of tests for *ndRSV* was done with samples of the primary endpoint-diluted *tdRSV*

stocks (Table 1); *ndRSV* was detected in none of the tests. These results indicate the spontaneous appearance of *ndRSV* during propagation of the secondary *tdRSV* stocks, and analysis of the proportion of secondary stocks from experiments FT105-1 and FT105-2 with no *ndRSV* (14, 16) estimates that from 0.16 to 0.64 transductions per culture had occurred. Kinetics of viral infection during propagation of secondary stocks were examined immunocytochemically (28) in duplicate, and an average was derived per culture of 2.6×10^6 cells which had been infected by virus (corrected for the number of infected cells which arose by division of previously infected cells). Under these conditions, cells infected early should contain a single provirus but later infections would be at high multiplicity. On average, one transduction occurred per 0.4×10^7 to 1.6×10^7 infected cells.

The transduction frequency with infecting virus is very low compared with that of homologous recombination between cellular sequences and DNA introduced by transfection or microinjection (1, 6, 31). In these cases, however, the very high frequencies reported may in part be attributed to site-specific linearization prior to introduction of DNA or to DNA damage resulting from the transfection procedure (2).

TABLE 3. Appearance of *ndRSV* in secondary *tdRSV* stocks^a

Test	Virus	Inoculum (μl)	No. of secondary stocks/total secondary stocks	
			<i>ndRSV</i>	No <i>ndRSV</i> (%)
FT101-1	<i>tdBerk101</i>	0.1	0/9	35/38 (92)
		1.0	2/10	
		10	0/9	
		100	1/10	
FT101-2a	<i>tdBerk101</i>	0.1	0/9	32/35 (91)
		1.0	1/10	
		10	0/7	
		100	2/9	
FT101-2b	<i>tdBerk101</i>	0.1	1/9	30/34 (88)
		1.0	1/10	
		10	0/7	
		100	2/8	
FT105-1	<i>tdBerk105</i>	0.1	3/20	17/20 (85)
FT105-2	<i>tdBerk105</i>	0.1	7/15	8/15 (53)

^a Secondary viral stocks were derived in a parallel series of cultures inoculated, as indicated, with primary endpoint-diluted *tdRSV* stocks. The secondary stocks were then tested for the presence of transforming virus by inoculating fresh CEF.

In addition, with transduction of *proto-src*, the recombination which is required to restore an isogenic *src* involves repair with *proto-src* sequences distributed over more than 5 kb; this reaction(s), by double crossover and asymmetric exchange, may have an inherently low probability of occurrence. Such recombination has been estimated to occur at low frequency between replication-deficient RSV, in non-producer quail cells, and infecting *tdRSV* (17). Repair of short gaps (<100 base pairs [bp]) in cloned, integration-deficient retrovirus mutants (24), presumably by recombination with endogenous proviral sequences (3), has been reported, but no attempt was made at quantitation. Homologous recombination by *tdRSV* in vitro with *proto-src* has previously been reported to occur at a low rate; viz., transformed foci appeared in later passages of infected cells, and variable deletion of 5' *src* sequences did not noticeably affect the rate as measured (again with the exception of NY18-3) (4); it would be of some interest to test these deletion mutants for transduction frequency under the conditions developed here.

The structure of three isolates of *ndRSV*, from independent transfections of *ptdBerk105* (*ndBerk105-1.1*, *ndBerk105-3.1*, and *ndBerk105-4.1*), and of several isolates from *tdBerk101* (*ndBerk101-2*, *ndBerk101-10.5*, and *ndBerk101-13.1*), as well as that of PrA RSV, was examined by Southern analysis of extrachromosomal viral DNA. A total of 23 cleavage sites, of six restriction endonucleases, was examined by hybridization with the *gag-pol-env* and *src* probes. As described above for *tdBerk105*, all digestions generated the fragments predicted by the physical maps (Fig. 2 and Table 2) and of the correct specificity with respect to the probe. Of particular interest are cleavage sites which may distinguish *proto-src* and PrA *src* alleles. Digestion of the three *ndRSV* DNAs with *Bgl*II resulted in two fragments, of 6.7 and 2.6 kb, homologous with the *gag-pol-env* probe (Fig. 1A, lanes 7, 15, and 20); only the 6.7-kb fragment is homologous with *src* (Fig. 1B, lanes 7, 15, and 20). In

contrast, the 6.7-kb fragment was not seen in PrA RSV DNA digested with *Bgl*II (Fig. 1A and B, lanes 2), while two smaller fragments of 3.5 and 3.2 kb and the 2.6-kb fragment were. (The two distinct bands of 3.5 and 3.2 kb seen in *Bgl*II-digested PrA RSV DNA indicate that the circular extrachromosomal viral DNA contains predominantly a single LTR, since two LTRs should result in two 3.5-kb fragments.) These results suggest that the internal *Bgl*II in the *src* of PrA is not present in the *ndRSV* DNA; this site is also absent from *proto-src* sequences (29). The *Bgl*II sites were mapped with respect to the unique *Not*I site in the LTR (Fig. 1A and B, lanes 2, 3, 7, 8, 15, 16, 20, and 21); the absence of the internal *Bgl*II site in *src* was also shown with respect to the *Mst*II sites in *src* (Fig. 1C, lanes 23, 24, 26, 27, 29, 30, 32, and 33). Analysis of the *src*-related fragments resistant to *Pst*I digestion of *ndRSV* DNA (Fig. 1B, lanes 9, 17, and 22; Fig. 1C, lanes 25, 28, and 31) and comparison with those of PrA RSV (Fig. 1B, lane 4; Fig. 1C, lane 34) indicate an additional *Pst*I site in the *ndRSV* allele, with a loss of one band of the 600-bp doublet, and an additional 344-bp band (visible most clearly in Fig. 1C). This additional *Pst*I site corresponds to the *proto-src* sequences in exon 6 (to which also those lacking the *Bgl*II site correspond). Thus, the presence of the *Bgl*II⁻ and *Pst*I⁺ markers in the *ndRSV* *src* allele is consistent with transduction of this central region from *proto-src*. The point of crossover in 5' *src* sequences was consistently in the 309-bp interval between the Δ *Hinc*II deletion and the upstream *Mst*II site, which is present in *tdBerk105* and all *ndRSV* (Fig. 1C, lanes 23, 26, and 29); the site is absent from *proto-src* (29) (confirmed by digestion of pCS3-BgK11, a subclone of λ CS-3 [25]; data not shown). Crossover in 3' *src* sequences must have occurred in the 144-bp interval between the Δ *Hinc*II deletion and the downstream *Pvu*II site present in *tdBerk105* and all *ndRSV* (Fig. 1C, lanes 38 to 41; the additional band of 1.2 kb in three samples is presumably from contaminating *tdRSV*). This *Pvu*II site is in a 36-bp region of *src* that is homologous with cellular sequences 900 bp downstream from the *proto-src* stop codon (29); evidence for recombination in these 3'-terminal sequences was not seen in any of the *ndRSV* isolates.

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