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Rescue of Chromosomal T-Antigen Sequences onto Extrachromosomally Replicating, Defective Simian Virus 40 DNA by Homologous Recombination

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Recombination between chromosomal and extrachromosomal DNA sequences was analyzed by investigation of the recombinational rescue of a 1,018-base-pair (bp) segment of the T-antigen gene of simian virus 40 from the chromosome of monkey COS cells to two different, extrachromosomally replicating, simian virus 40 DNA molecules lacking this 1,018-bp sequence. The ratio of rescued to unrecombined virus was as high as 10^{-3}. The rescued molecules, detected optimally 5 to 9 days after transfection of COS cells, had completely recovered the 1,018-bp DNA segment from the chromosome. The recombination event is proposed to occur either by double reciprocal recombination or by gene conversion between the chromosomal T-antigen gene and the extrachromosomal molecules missing the 1,018-bp sequence.

Even though mitotically dividing mammalian cells exhibit a variety of recombinational activities (2, 5, 9–13, 18–24), the targeting of genes to specific chromosomal locations by homologous recombination is a rare and inefficient process (12, 22, 23). In contrast, the integration of plasmids into homologous regions of the chromosome is readily observed in *Saccharomyces cerevisiae* yeast (7, 16). Ongoing research in a number of laboratories is therefore focused on the development of suitable selection schemes to detect rare targeting events in mammalian cells. It is equally important, however, to develop procedures for the rescue of chromosomal alleles onto extrachromosomal molecules, as this would allow the rapid isolation and mapping of interesting chromosomal mutations, as has been demonstrated with yeast (17).

A 1,018-base-pair (bp) DNA segment was excised from the early regions of either wild-type or a viable deletion mutant of simian virus 40 (SV40). Introduction of these linear, gapped DNAs independently into monkey COS cells, which contain an integrated copy of the SV40 early region (6), resulted in the rescue of the missing genetic information from the chromosome. The frequencies, the time course, and the products of these recombination events were characterized. The 1,018-bp DNA sequence (which includes parts of the large T- and small t-antigen coding regions and the intron from the small t-antigen gene) was rescued completely and accurately from the chromosome in all of the products analyzed.

SV40 DNA has two *Nde*I sites (Fig. 1A) at nucleotides (nt) 4826 and 3808 in the early region (1). Since the large 4,225-bp *Nde*I fragment from SV40 was needed free of the 1,018-bp segment, the cloning scheme shown in Fig. 1A was undertaken. The 4,225-bp *Nde*I fragment from SV40 was purified and inserted into the unique *Nde*I site (nt 2297) in pBR322 DNA to obtain pSVN. The large 4,189-bp *Nde*I fragment from *dl*1262, a viable deletion mutant of SV40 lacking nt 1319 to 1354 (3), was also cloned independently into pBR322 to obtain p1262N, as shown in Fig. 1A.

Experimental strategy to detect rescue. In studying the rescue of chromosomal information onto an extrachromosomal molecule, it is necessary not only to detect the event but also to measure the frequency of generation of rescued molecules. The protocol used is shown in Fig. 1B. DNA from pSVN or p1262N was digested completely with *Nde*I to release the SV40 deletion from pBR322. Since the SV40 and pBR322 DNA fragments were similar in size they were used as a mixture. The linear, *Nde*I-digested SV40 deletion was transfected into COS cells (COS-1) on a series of plates (80% confluent with about 5×10^6 cells per plate) by the DEAE-dextran procedure (14). Each 10-cm-diameter dish was transfected with 400 ng of *Nde*I-digested pSVN or p1262N DNA containing 200 ng of the SV40 deletion. For the transfection, each plate of cells was washed twice with Tris-saline, incubated at room temperature for 15 min with 0.5 ml of DNA containing 500 μg of DEAE-dextran per ml, and then washed twice with Tris-saline before culture medium containing 2% fetal calf serum was added. The plates were then placed at 37°C in a CO_2 incubator.

Linear DNA was used to transfect COS cells because my earlier studies had indicated that double-strand breaks within the region of homology stimulated the frequency of extrachromosomal recombination in COS cells 10- to 50-fold relative to supercoiled DNA (18). Once the linear DNA enters the nuclei of transfected cells, some of it could circularize by ligation of the ends (i.e., no recombination occurs). Both linear and circular defective SV40 molecules could interact with the homologous T-antigen gene in the chromosome as a prelude to the rescue event. Complete rescue of the missing 1,018-bp segment of the T-antigen gene from the chromosome results in the generation of wild-type SV40 which replicates and is amplified by reinfection of other cells. The circular, unrecombined molecules containing the 1,018-bp deletion can also replicate in COS cells and be packaged into virions, which can then be released to infect other cells not infected during the initial transfection procedure. In these virus-infected cells, further recombination could occur between circular SV40 molecules with the deletion and the chromosomal T-antigen gene to generate wild-type SV40. Thus, both the recombination substrate and product are amplified by replication and viral spread through all the cells in the population, making it easier to detect rare events. Furthermore, there is no specific selection for the recombinant molecules in COS cells.

Timing of rescue. To follow the timing of the events, virus

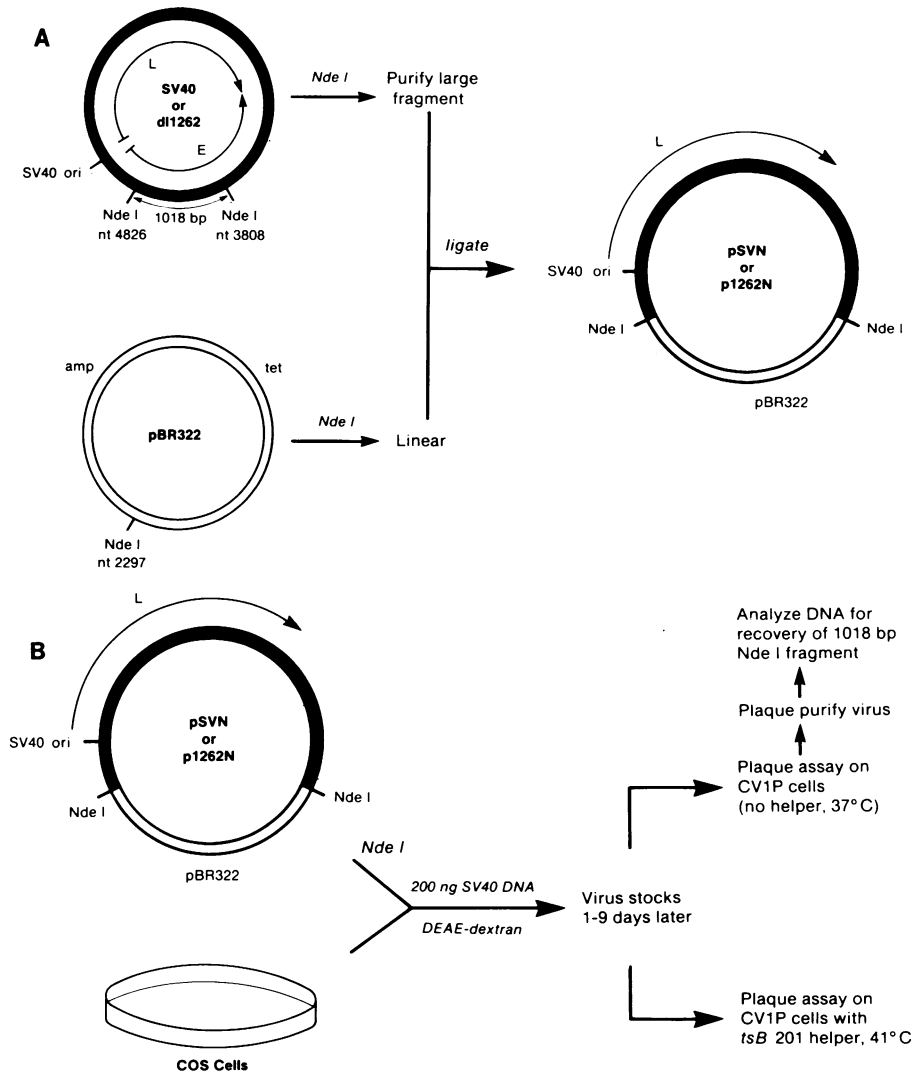

FIG. 1. Construction of pSVN and p1262N and the scheme for detection and quantitation of rescued molecules. (A) DNA from wild-type SV40 or *dl*1262 (a viable deletion mutant of SV40) was digested with *Nde*I. The large *Nde*I fragment was purified and inserted into the unique *Nde*I site in pBR322. pSVN and p1262N denote the *Nde*I deletions of SV40 and *dl*1262, respectively. (B) SV40 or *dl*1262 DNA lacking a 1,018-bp *Nde*I fragment from the early region was transfected into monkey COS cells containing an integrated copy of the SV40 early region. Recombinational rescue restores the deleted DNA in the input molecules and results in the production of virus particles capable of forming plaques on CV1P cells at 37°C in the absence of helper virus. Unrecombined molecules can circularize and also be packaged into virions. Titers of these are determined by plaque assay on CV1P cells with *tsB*201 helper virus at 41°C. The ratio of titers obtained in the absence and presence of helper virus gives the frequency of rescued molecules. The late (L) and early (E) regions of SV40 are also shown.

stocks (1 ml) were made 1 to 9 days after transfection, with the medium being changed once after 4 to 5 days. By the end of this period, the COS cells showed extensive cytopathic effect. Titers for the rescued virus in each of these virus stocks were determined by plaque assays (14) on monkey CV1P cells (no helper, 37°C) as shown in Fig. 1B. Viruses that had not acquired the 1,018-bp DNA segment constitute the unrecombined population. They also multiply in the COS cells since they are complemented for growth by the T-antigen synthesized by these cells. Titers for this population, defective in the early region, were determined on CV1P cells at 41°C with a helper virus carrying a temperature-sensitive mutation in the SV40 late region (*tsB*201, 10^5 PFU per 60-mm-diameter plate). All virus titers were obtained from slopes of graphs in which the number of plaques produced by a series of virus dilutions was plotted. The ratio of the virus titers in the absence and presence of the helper virus gave the frequency of rescued molecules.

Rescued virus could be detected typically 5 to 9 days after transfection, and the frequency of rescued molecules was as high as 10^{-3} (Table 1). The time course, however, was not perfectly reproducible from experiment to experiment, as shown in Table 1. This is not too surprising because the frequency at each time point depends on when the rescue occurred. If the recombination event occurred early in that plate of cells, then the frequency of rescue was high for that time point. Conversely, if the event occurred late, then the frequency was low for that time point. However, in seven independent time-course experiments, wild-type virus was always rescued from one or more of the time points between 5 and 9 days.

To determine whether wild-type SV40 had any replicative

TABLE 1. Frequencies of rescued SV40 molecules[a]

Time after transfection of COS cells (days)	Results with *Nde*I deletion of wild-type SV40 in expt: 1			2			3			Results with *Nde*I deletion of *dl*1262 in expt 4		
	Amt of virus rescued (PFU/ml):		Frequency of rescued molecules	Amt of virus rescued (PFU/ml):		Frequency of rescued molecules	Amt of virus rescued (PFU/ml):		Frequency of rescued molecules	Amt of virus rescued (PFU/ml):		Frequency of rescued molecules
	Without helper at 37°C	With helper at 41°C		Without helper at 37°C	With helper at 41°C		Without helper at 37°C	With helper at 41°C		Without helper at 37°C	With helper at 41°C	
1–3	0		—[b]	0		—	0		—	0		—
4	0		—	0		—	0		—	375	1.2×10^5	3.1×10^{-3}
5	10	6.6×10^5	1.5×10^{-5}	0		—	0		—	5	2.6×10^6	1.9×10^{-6}
6	80	5.4×10^6	1.5×10^{-5}	5.7×10^5	4.3×10^8	1.3×10^{-3}	100	2.4×10^5	4.1×10^{-4}	250	6.6×10^6	3.8×10^{-5}
7	4.7×10^4	2.6×10^7	1.8×10^{-3}	9.1×10^3	3.0×10^8	3.0×10^{-5}	0		—	0		—
8	6.3×10^2	5.9×10^7	1.1×10^{-5}	3.7×10^3	1.6×10^8	2.3×10^{-5}	100	3.1×10^5	3.2×10^{-4}	0		—
9	7.5×10^2	2.3×10^7	3.3×10^{-5}	4.4×10^2	1.2×10^8	3.7×10^{-6}	0		—	350	1.4×10^7	2.5×10^{-5}

[a] Titers of virus stocks (1 ml) obtained 1 to 9 days after transfection were determined on CV1P cells in the absence and presence of *tsB*201 to obtain the frequency of rescued molecules, which is the ratio of rescued to unrecombined virus, as described in the text. Mock infections produced no plaques either with or without helper virus. The titer of virus from one plate was determined for each time point in each experiment.

[b] —, No rescued molecules were detected.

advantage over the *Nde*I deletions used, SV40 DNA linearized with *Bgl*I was cotransfected into COS cells along with *Nde*I-digested, linear pSVN or p1262N DNAs at different ratios. Seven days later, Hirt DNAs were prepared, linearized with *Bgl*I, electrophoresed on agarose gels, transferred to nitrocellulose, and probed with nick-translated SV40 DNA. SV40 had only a marginally higher growth rate and was present in only sixfold higher amounts than those expected from the ratios of the input DNAs (Table 2). When one considers the multiple rounds of viral replication possible in 7 days and the large number of virus particles released after each infectious cycle, this sixfold higher amount of SV40 DNA translates into a very small growth advantage per replication cycle. Thus, the data obtained between 7 and 9 days for the frequency of rescued molecules (Table 1) could not have been overestimated by more than a factor of 10.

Controls. A number of control experiments were done to ensure that this rescue was not due to artifactual contamination with SV40. Firstly, each batch of DNA used to transfect COS cells was tested directly on CV1P cells. No plaques were ever obtained. This rules out contamination of the DNA and other solutions. Secondly, virus stocks made from mock-infected COS plates never produced any plaques on CV1P cells, either in the absence or in the presence of the helper, even when the entire virus stock from a COS plate was used. This eliminates contamination of the COS cells or the culture medium with SV40. Mock infections of the CV1P cells were also done when titers of each virus stock were determined. Again, no plaques were seen. Finally, as described later, COS cells transfected with the *Nde*I deletion of a physically marked SV40 mutant (*dl*1262) generated the corresponding rescued virus.

TABLE 2. Relative replication abilities of SV40 and its *Nde*I deletion mutants in COS cells[a]

Input DNA ratio		([SV40/*Nde*I Δ] observed)/ ([SV40/*Nde*I Δ] expected)[b]
SV40:*Nde*I Δ of SV40	SV40:*Nde*I Δ of *dl*1262	
1:1		3.6
3:1		4.1
4:1		8.9
	3:1	8.6
	4:1	4.6

[a] SV40 and its *Nde*I deletion (Δ) mutants were cotransfected into COS cells as linear DNAs at the ratios indicated, keeping the total amount of viral DNA constant at 200 ng per 10-cm plate. Hirt DNAs obtained 7 days posttransfection were linearized and subjected to Southern blot analysis as described in the text. The quantitation was done by densitometric scanning of the resulting autoradiograms.

[b] Average was six.

Analysis of rescued products. A total of 37 plaques derived from seven independent transfections of COS cells were picked from the CV1P plates grown at 37°C in the absence of helper. These were plaque purified, and the low-titer virus from these plaques was used to infect CV1 cells for DNA. Two weeks later, the DNAs were isolated by the Hirt procedure (8), followed by purification on CsCl-ethidium bromide density gradients. These DNAs were then digested with a number of enzymes and compared with DNA from wild-type SV40. All 37 DNAs were identical to DNA from wild-type SV40, as shown by the representative set of digests in Fig. 2. The *Hin*dIII digests in Fig. 2A of six of the rescued DNAs (lanes 1 to 6) were identical to that of DNA from wild-type SV40 (lane 7). All 37 DNAs had also rescued the 1,018-bp DNA segment from the COS cell chromosome and regenerated precisely the two *Nde*I sites flanking this sequence, as shown for some of the DNAs in Fig. 2B.

Rescued molecules are related to input DNA. In addition to precautions taken to ensure that contamination was not the source of the rescued virus, the recombination event was documented more rigorously with *dl*1262, a viable deletion mutant of SV40 carrying a 36-bp deletion (3) that physically distinguishes the late region of this mutant from that of SV40. We have never worked with *dl*1262 virus before and hence the likelihood of contamination with *dl*1262 virus was nonexistent. When the *Nde*I deletion of *dl*1262 was introduced into COS cells, recombinant virus was rescued between 5 and 9 days (Table 1). In this case also, the ratio of rescued to unrecombined virus was as high as 10^{-3}.

From the experiments in which the *Nde*I deletion of *dl*1262 was used, 13 plaques derived from four independent transfections of COS cells were picked, the virus was plaque purified, and DNAs obtained with these virus stocks were analyzed as described earlier. In every case, the rescued virus was identical to *dl*1262 DNA, as illustrated by the *Hin*dIII digests in Fig. 3A. Plasmid p1262N (Fig. 3A, lane 8), lacks the 1,169-bp and 526-bp *Hin*dIII fragments originating from the SV40 early region (Fig. 3A, lane 1) because of the deleted 1,018-bp DNA segment. In addition, the 447-bp *Hin*dIII fragment derived from nt 1046 to 1493 of the SV40

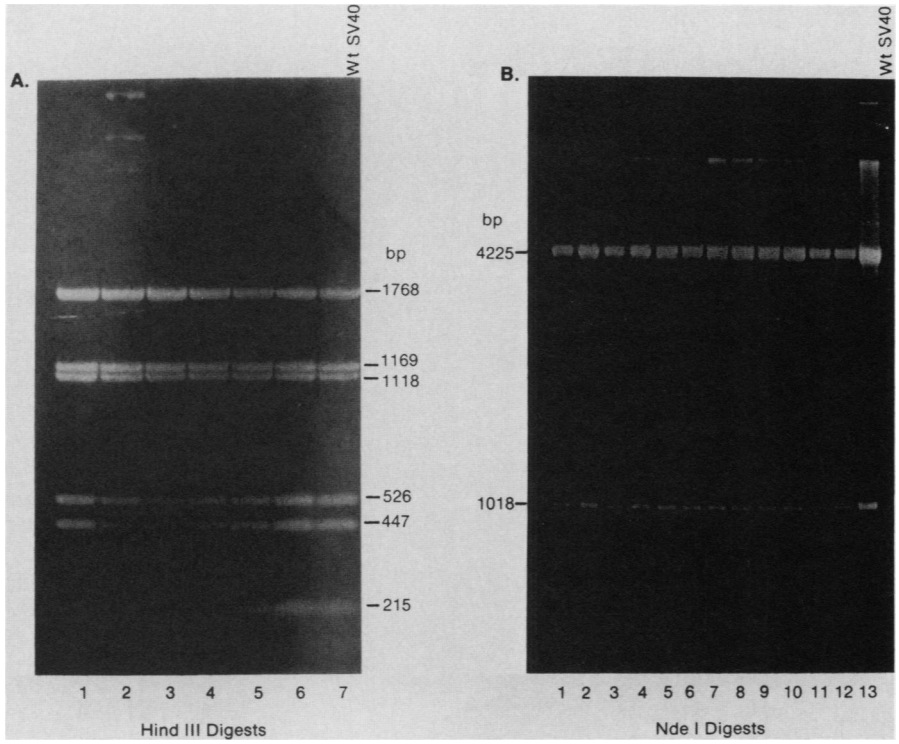

FIG. 2. Proof of recombinational rescue for SV40. Passage of the *Nde*I deletion of SV40 through COS cells generated rescued virus. DNAs from a number of independently derived stocks of rescued virus were digested with restriction endonucleases and compared with wild-type SV40. (A) *Hin*dIII digests of rescued viruses (lanes 1 to 6) and wild-type (Wt) SV40 (lane 7). Fragment sizes are on the right. (B) *Nde*I digests of rescued viruses (lanes 1 to 12) and wild-type (Wt) SV40 (lane 13). Fragment sizes are on the left.

late region is smaller by 36 bp in p1262N (Fig. 3A, lane 8). All the rescued DNAs had regained the 1169- and 526-bp *Hin*dIII fragments but also contained the 411-bp fragment diagnostic of *dl*1262. In addition, (Fig. 3B) all these DNAs had acquired the 1,018-bp *Nde*I fragment. This shows that the recombination product is derived from the recombination substrate, as expected. Furthermore, since the 36-bp deletion in the late region of *dl*1262 was 1.74 and 2.45 kilobases from the endpoints of the 1,018-bp deletion, it is not too surprising that no coconversion of the 36-bp deletion was observed in the products analyzed.

It should be noted that the 50 products analyzed (37 with SV40 and 13 with *dl*1262) were derived from 11 independent transfections of COS cells. In every case, the missing 1,018-bp deletion had been completely and faithfully restored with the regeneration of both the *Nde*I sites flanking the deletion. The information transferred from the chromosome includes parts of the small t- and large T-antigen-coding sequences and the intron in the small t-antigen gene. One boundary of the deletion that is corrected (*Nde*I site at nt 4826 in SV40) is 91 and 254 nt away from the splice donor and acceptor sites, respectively, in the intron for the T-antigen gene (1). Since deletion mutants with endpoints within these intron boundaries are viable (4), some of the products could have shown incomplete rescue of this information and still have produced plaques. Yet none of the 50 products showed partial rescue of the 1,018-bp sequence. This is unlike the situation in yeast in which partial repair, or deletions extending beyond the boundary of the double-strand gap, have been seen in about 20% of the molecules in which there was no selection for complete repair (15).

The COS-1 cells used in these experiments contain only one integrated copy of the SV40 T-antigen gene and lack most of the late region sequences (6). Since Gluzman (6) and I have observed that COS cells do not contain the T-antigen gene in the extrachromosomal state or produce SV40 virus by excision from integrated sequences (footnote *a* Table 1), it is likely that the rescue of the 1,018-bp DNA segment occurs from the chromosome onto the defective SV40 molecules.

Though the COS cells were transfected with linear DNA with the hope that double-strand breaks would stimulate recombination, the observation that the rescued virus appears primarily between 5 and 9 days after transfection suggests that the linear DNA itself may not be contributing much to the high recombination frequency except perhaps in the few plates which produce exceptionally high frequencies (10^{-3}) of rescued molecules. It seems more likely that the feature that makes this assay work efficiently in detecting what might otherwise be a rare event is the ability of the deleted SV40 substrate to circularize, replicate, and be spread from cell to cell. If one views the time points in Table 1 as independent samples of a fluctuation test, one can calculate, based on the observation that 30% of the plates do not yield virus between 5 and 9 days, that the recombination event probably occurs in 1 of about 10^7 infected COS cells. This is generally consistent with the frequency of gene targeting (12, 22, 23). It should also be emphasized that the frequency of rescued molecules depends on the timing of the recombination event and should not be confused with the fact that the recombination event occurs in only 1 of approximately 10^7 cells.

Possible mechanisms. The recombinational rescue could have occurred either by a double reciprocal crossover event

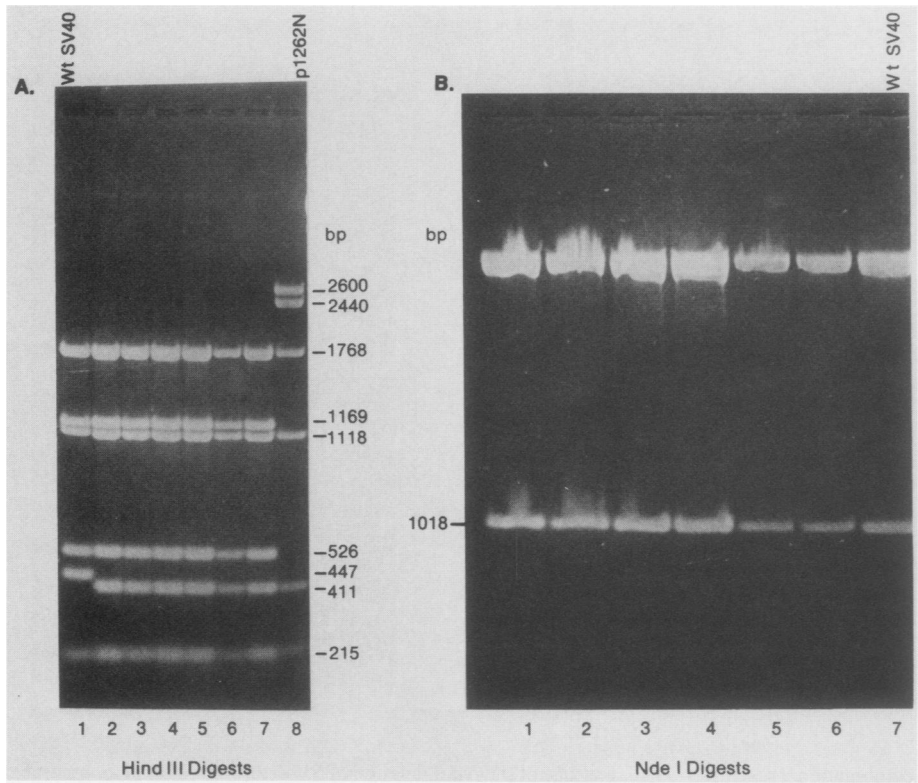

FIG. 3. Proof of recombinational rescue for *dl*1262. Passage of the *Nde*I deletion of *dl*1262 through COS cells generated rescued virus. DNAs from a number of stocks of rescued virus were digested with restriction enzymes and compared with wild-type SV40 and p1262N DNA. (A) *Hin*dIII digests of wild-type (Wt) SV40 (lane 1), rescued viruses (lanes 2 to 7), and p1262N (lane 8). Fragment sizes are on the right. (B) *Nde*I digests of rescued viruses (lanes 1 to 6) and wild-type (Wt) SV40 (lane 7).

or by nonreciprocal gene conversion from the chromosome onto the deleted SV40. Two classes of double crossover events are plausible. In the first, homologous integration of the deleted SV40 could occur to create a tandem duplication of the wild-type and mutant T-antigen genes in the chromosome, followed by excision of wild-type SV40. In the second type of event, a simultaneous double crossover could occur between the deleted SV40 (linear or circular) and the chromosome such that the exchanges occur in the regions of homology flanking the gapped DNA. Both of these reciprocal recombination mechanisms would result in the transplacement of the wild-type T-antigen gene in the chromosome with the deleted T-antigen gene from the incoming SV40 DNA. Alternatively, nonreciprocal gene conversion from the chromosome to the extachromosomal molecule could occur, leaving a wild-type T-antigen gene on both chromosomal and extrachromosomal DNAs. These mechanisms or the double-strand break repair model proposed for yeast (25) could explain the results, but further experiments are necessary to distinguish which of these mechanisms is correct. The wild-type virus detected by Gluzman (6) and Vogel et al. (26) upon passage of either deletion mutants of the SV40 early region in COS cells or temperature-sensitive SV40 mutants through SV40-transformed monkey cells may have been produced in a similar fashion. Whatever the mechanism, it seems clear that extrachromosomal DNA can find homologous chromosomal sequences and interact with them. In this work, only the rescued extrachromosomal molecules were analyzed, but the results suggest that the targeting of genes should also be detectable.

Recently, Shaul et al. (20) published a report on the generation of wild-type SV40 from COS cells infected with SV40 carrying the human insulin gene in the early region. Their results also provide evidence for recombinational rescue, but the exact frequencies, time course, and characterization of large numbers of independently derived products were not described in their paper.

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