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Mapping of Alterations in Noninfectious Proviruses of Spleen Necrosis Virus

JULIAN J. O'REAR AND HOWARD M. TEMIN*

Department of Oncology, University of Wisconsin Medical School, Madison, Wisconsin 53706

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Ten recombinant lambda phage containing proviruses of spleen necrosis virus (SNV) were previously obtained. Six of the proviruses are infectious and four are not infectious in infectious DNA assays. In this paper, we show that these noninfectious proviruses are not infectious because of alterations in the viral DNA. We constructed recombinants between infectious and noninfectious proviruses and tested these recombinants in an infectious DNA assay. In addition, we carried out cotransfection of a noninfectious provirus with a restriction endonuclease-generated fragment of viral DNA. The alterations in the viral DNA resulting in lack of infectivity were mapped to regions of viral DNA of 1 to 2 kilobase pairs. These results and other biochemical data indicate that alterations in retrovirus proviruses occur at a high frequency.

Infection of chicken embryo fibroblasts with spleen necosis virus (SNV) occurs in two phases. The acute phase, which lasts from approximately 2 to 10 days after infection, is characterized by a strong cytopathic effect (26). This phase is followed by the chronic phase, in which the cytopathic effect disappears (27). Virus production occurs during both phases. If DNA from chicken cells acutely infected with SNV is digested with restriction endonuclease *Eco*RI, which does not cleave SNV DNA, and is fractionated on an agarose gel, infectious fragments of many sizes are found (2). In contrast, infectious fragments of a limited size range are produced when DNA from chronically infected cells is digested with restriction endonuclease *Eco*RI and is fractionated on an agarose gel. These results seemed to indicate a "unique" site of integration of infectious DNA in chronically infected cells (2). However, when integrated viral DNA is detected by nucleic acid hybridization, the fragments containing viral DNA are heterogeneous in size in both acutely and chronically infected cells (16). The results of this combination of nucleic acid hybridization and infectivity experiments indicate that some viral DNA sequences in the *Eco*RI-generated fragments larger and smaller than the infectious fragments from chronically infected cells are not expressed in an infectious DNA assay.

The correlation of the size of *Eco*RI-generated fragments and infectivity could be a result of insertions, deletions, or rearrangements in viral DNA integrated at other than a unique site in cellular DNA. However, the majority of the noninfectious viral DNA has no gross alterations of the viral DNA as determined by restriction enzyme analysis (15). Other explanations for the lack of infectivity, which do not account for the correlation of size with infectivity, are minor alterations in the viral DNA sequences or the influence on virus expression of flanking cellular DNA sequences. To distinguish between these two possibilities and to look for explanations of the correlation of size with infectivity, we obtained recombinant DNA clones containing infectious and noninfectious proviruses (19). Although the infectious proviruses were integrated in *Eco*R1 fragments of a similar size, the proviruses were not integrated at a unique site. Furthermore, none of the recombinant DNA clones containing noninfectious proviruses had major alterations in the viral sequences.

These clones containing proviruses have been used to develop techniques for mapping the sites affecting infectivity. We have mapped these sites by the in vitro construction of recombinants between infectious and noninfectious proviruses and by virus recovery after cotransfections of noninfectious proviruses and cloned fragments of SNV DNA. These biological assays detect alterations in the four noninfectious proviruses which were not apparent in restriction enzyme analyses. However, the correlation of infectivity with the size of those fragments generated with the restriction endonuclease *Eco*RI which contain viral DNA is still unexplained.

MATERIALS AND METHODS

Cells and viruses. The procedures for obtaining and propagating avian cells and avian reticuloendotheliosis viruses have been described (13, 26, 27).

Preparation of phage DNAs. Growth and purification of phage have been described previously (19). DNA was purified by phenol extraction.

DNA transfections. DNA transfections were done according to a modification (8) of the procedure of Graham and van der Eb (11). The production of virus was detected by cytopathic effects. Virus recovered at the endpoint of cotransfections was quantitated by endpoint dilution assay and detected by cytopathic effects (26).

Digestion of DNA with restriction endonucleases. Restriction endonucleases *Bgl*II, *Sac*I, *Sal*I, and *Xho*I were purchased from New England Biolabs and used with the appropriate buffer indicated by the supplier. Restriction endonuclease *Eco*RI was purified in out laboratory and used with 10 mM Tris (pH 7.4), 10 mM $MgCl_2$, 10 mM dithiothreitol, 200 mM NaCl, and 100 μg of bovine serum albumin per ml.

Preparation of nick-translated SNV [^{32}P]DNA. The 7.7-kilobase pair (kbp) *Sal*I fragment of clone 60B was subcloned into the *Sal*I site in pBR322 as described before (24). This plasmid was nick translated by a modification of the procedure of Rigby et al. (21) with [α-^{32}P]dCTP (>300 Ci/mmol; Amersham Searle).

Construction of recombinants. Equimolar amounts of DNA from an infectious and a noninfectious clone were mixed. The DNA was digested with a restriction endonuclease which cleaves viral DNA once and does not cleave Charon 4A DNA or the chicken DNA. The DNA was ligated (22) and then packaged in vitro (5). The resultant phage were plated on DP50supF, and plaques were screened by digesting DNA prepared from plate lysates with restriction endonucleases.

Preparation of DNA from plate lysates. A well-isolated plaque was picked and suspended in 10 mM Tris-hydrochloride (pH 7.4), 10 mM NaCl, 10 mM $MgCl_2$, and 0.05% (wt/vol) gelatin. One hundred microliters of an overnight culture of DP50supF was infected with approximately 10^5 phage and plated on a 100-mm plate in NZYDT top agar made with 1.5% (wt/vol) sea plaque agarose (Marine Colloids, Rockland, Maine). This infection produced confluent lysis of the bacterial lawn. The plate was cooled, and 2 ml of cold 10 mM Tris-hydrochloride (pH 7.4)–10 mM $MgCl_2$ was overlaid and allowed to sit at 4°C overnight. The lysate was cleared by adding a drop of chloroform. Then 0.4 ml of the cleared lysate was pipetted into a 1.5-ml microfuge tube. Two microliters of diethylpyrocarbonate, 8 μl of 20% sodium dodecyl sulfate, and 100 μl of 2 M Tris-hydrochloride–0.2 M EDTA (pH 8.5) were added with mixing. The lysate was then incubated at 67°C for 10 min in a hood. After the lysate was cooled on ice, 50 μl of cold 5 M sodium acetate was added, and the lysate was incubated on ice for 30 min. The precipitate was removed by centrifugation in a microfuge for 15 min. The supernatant was transferred to another microfuge tube, and the tube was filled with 95% ethanol at room temperature. After 5 min at room temperature, the precipitate was pelleted in the microfuge by centrifuging for 5 min. The supernatant was discarded, and the pellet was allowed to dry. The precipitated DNA was dissolved in 50 μl of 10 mM Tris-hydrochloride (pH 8.0), 1 mM EDTA, and 1 μg of RNase A per ml. After incubation for 60 min at 37°C, 1 μl of diethylpyrocarbonate was added. The DNA was left at room temperature overnight to destroy the diethylpyrocarbonate. Ten microliters of the DNA solution was used for each digestion.

Minipreps of plasmid DNA. For screening of plasmids, minipreps were done according to a procedure of W. Cipriano (unpublished data). The colony to be tested was picked with a sterile toothpick and inoculated into 1 ml of L broth in a 1.5-ml Eppendorf tube. The tube was covered with foil and incubated at 37°C overnight without shaking. Cells were pelleted in a Beckman microfuge and suspended, using a Vortex mixer, in 70 μl of 50 mM Tris-hydrochloride (pH 8.0), 50 mM NaCl, and 62.5 mM EDTA. Fifteen microliters of 10 mM Tris-hydrochloride (pH 7.3)–14 mM NaCl containing lysozyme (3 mg/ml) and RNase A (2 mg/ml) was added and mixed with the aid of a Vortex mixer. The cells were incubated 10 min on ice and lysed with heat, 5 min at 90°C or 3 min at 95°C. Debris was pelleted in the microfuge by centrifugation for 15 min. One drop of phenol was added to the supernatant, an emulsion was made by using a Vortex mixer, and the tube was centrifuged. The aqueous phase was extracted with chloroform and ethanol precipitated. The pellet was suspended in 20 μl of 250 mM lithium acetate–5 mM $MgCl_2$ and ethanol precipitated again. The pellet was suspended in 20 μl of double-distilled water. Ten microliters of this solution was used in digestions.

RESULTS

Construction in vitro of recombinants. Infectious and noninfectious proviruses were cloned in the *Eco*RI site of Charon 4A (19). Figure 1 shows restriction endonuclease cleavage maps of the infectious and noninfectious proviruses used in these experiments.

To map in the cloned DNA the site(s) affecting virus expression, we constructed in vitro recombinants of infectious and noninfectious proviruses. Restriction endonucleases *Sal*I and *Xho*I cleave SNV DNA once (15) and do not cleave Charon 4A DNA (9). Recombinants were constructed by using *Sal*I and *Xho*I in those cases in which these enzymes did not cleave the cellular sequences adjacent to the noninfectious provirus.

Recombinants between clone 14-44, containing an infectious provirus (>10^4 IU/μg), and clone 3-73, containing a noninfectious provirus (<1 IU/5 μg), were constructed with *Xho*I and with *Sal*I. The structure of the recombinants was tested by digestion with *Xho*I and *Eco*RI followed by electrophoresis through an agarose gel. Figure 2A shows an example of the recombinants, in this case constructed with *Xho*I, which cleaved 60% from the 5′ end of the provirus. The left slot contained DNA from clone 14-44, which had an infectious provirus, and the right slot contained DNA from clone 3-73, which had a noninfectious provirus. The recombinant

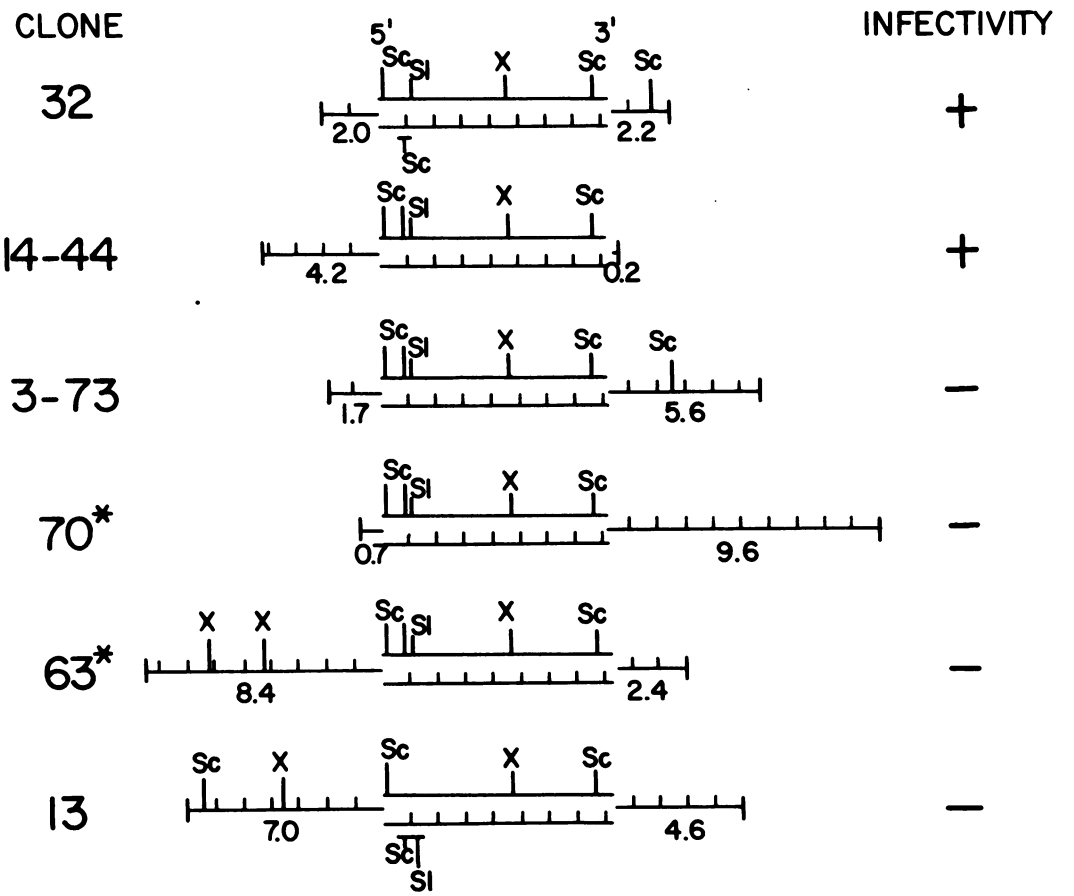


FIG. 1. *Maps of the cleavage sites of SacI, SalI, and XhoI in the inserted fragments of Charon 4A clones containing SNV proviruses. Double lines indicate viral sequences, 8.3 kbp in size, and a single line indicates cellular sequences. T indicates a restriction enzyme cleavage site in viral DNA that is not present in that provirus. Vertical bars are separated by 1 kbp. The sizes of the flanking cellular sequences appear under the cellular sequences and are given in kilobase pairs. Symbols: (+) >10^4 IU per μg of clone DNA; (−) <1 IU per 5 μg of DNA; (*) clones in which the SacI cleavage sites in the cellular DNA have not been mapped. The orientation of the EcoRI fragment in clone 70 was changed for these studies. 5′ and 3′ denote the orientation of the provirus with respect to the viral RNA. Abrreviations: Sc, SacI; SI, SalI; X, XhoI.*

in the second slot from the left had the 5′ end of clone 3-73 and the 3′ end of clone 14-44. The third slot from the left contained the reciprocal recombinant, the 5′ end of clone 14-44, and the 3′ end of clone 3-73. When these recombinants were tested for infectivity, the recombinant with the 5′ end of clone 3-73 was not infectious, but the reciprocal recombinant was. This result showed that the site affecting infectivity was in the viral sequences to the 5′ side of the *Xho*I site or in the cellular sequences of clone 3-73 at the 5′ end of the provirus. When the recombinants were generated by using *Sal*I, which cleaved 10% from the 5′ end of the provirus, the recombinant with the 3′ end of clone 3-73 was not infectious, but the reciprocal recombinant was. The only difference between the two sets of recombinant proviruses was a difference in the viral DNA between the *Sal*I and *Xho*I cleavage sites. Therefore, the site affecting infectivity mapped in the viral DNA of clone 3-73 between the *Sal*I and the *Xho*I sites.

Figure 2B shows recombinants, constructed with *Sal*I, between clone 14-44, containing an infectious provirus, and clone 63, containing a noninfectious provirus. DNAs from the recombinants were digested with *Xho*I and subjected to electrophoresis through an agarose gel. The first recombinant (slot 2) had the 5′ end of clone 63 and the 3′ end of clone 14-44, and the reciprocal recombinant (slot 3) had the 5′ end of clone 14-44 and the 3′ end of clone 63. The recombi-

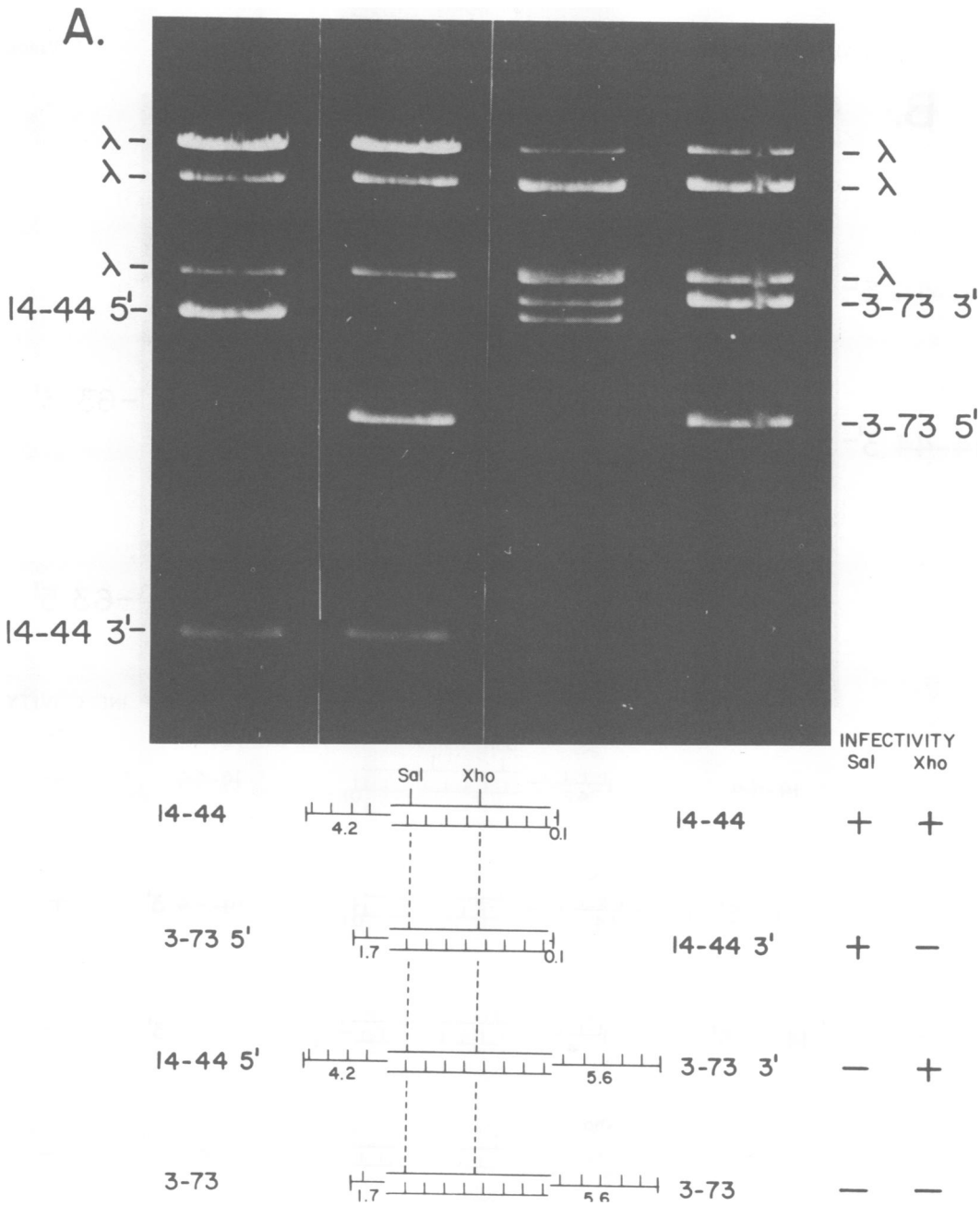


FIG. 2. *Recombinants of infectious and noninfectious proviruses. One-half microgram of DNA from parental and recombinant phages was digested with the indicated restriction enzyme(s) and was subjected to electrophoresis through a 0.7% (wt/vol) agarose gel containing 0.5 μg of ethidium bromide per ml (23). The left lane in each gel has DNA from the parental phage containing an infectious provirus; the right lane has DNA from the parental phage containing a noninfectious provirus. The second lane from the left contains DNA from phage with the recombinant provirus having the 5' end of the noninfectious provirus and the 3' end of the infectious provirus. The third lane contains DNA from phage having the reciprocal recombinant. Below each gel are maps of the parental and recombinant proviruses and their adjacent cellular DNA as well as the results of infectious DNA assays. Double lines indicate viral sequences, and a single line indicates cellular sequences. Vertical bars are separated by 1 kbp, and numbers under the cellular sequences give the size in kilobase pairs. Symbols: (+) $>10^4$ IU per μg of clone DNA; (–) <1 IU per μg of clone DNA. (A) DNAs from clones 14-44 and 3-73 and their recombinants constructed with XhoI were digested with EcoRI and XhoI. (B) DNAs from clones 14-44 and 63 and their recombinants constructed with SalI were digested with XhoI. (C) DNAs from clones 32 and 70 and their recombinants constructed with SalI were digested with EcoRI and XhoI. The faint band of DNA at 12 kbp in slot 3 and 10.5 kbp in slot 4 is the EcoRI fragment of DNA from phage that have lost most of the SNV sequences by intermolecular or intramolecular recombination between the long terminal repeats (19).*

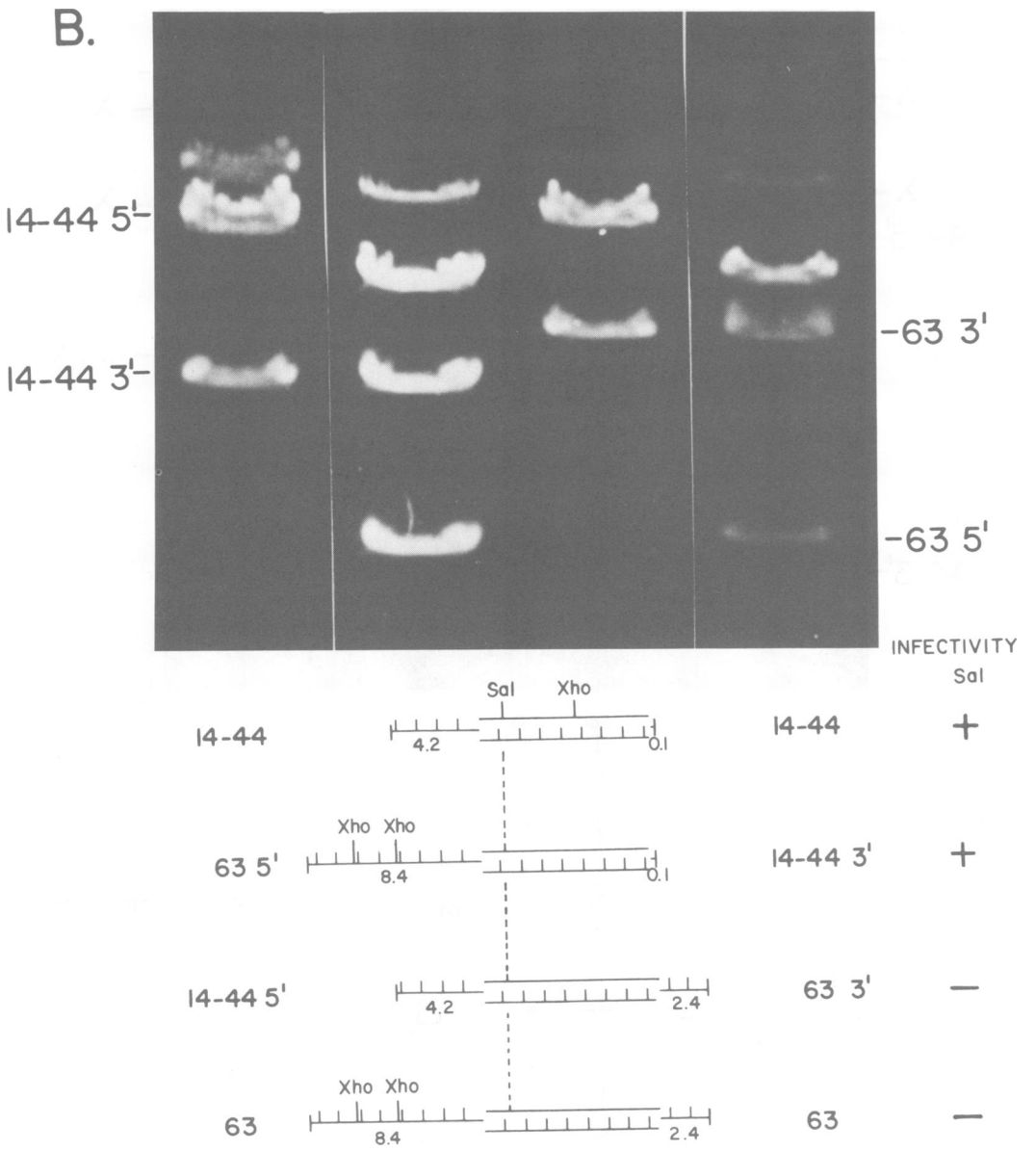


FIG. 2—*Continued*

nant with the 5′ end of clone 63 and the 3′ end of clone 14-44 was infectious; the reciprocal recombinant was not. These results map the site affecting infectivity in the 3′ 90% of the viral sequences or in the cellular sequences of clone 63 at the 3′ end of the provirus. The infectious recombinant was larger than the noninfectious recombinant, 16.8 kbp compared with 14.9 kbp. This size comparison supports our previous conclusion that infectivity is not a function of size alone (2, 19).

Figure 2C shows DNA from recombinants, constructed with *Sal*I, between clone 32, containing an infectious provirus, and clone 70, containing a noninfectious provirus. The DNAs were digested with *Xho*I and *Eco*RI and were subjected to electrophoresis through an agarose gel. The first recombinant (slot 2) had the 5′ end of clone 70 and the 3′ end of clone 32. The third slot contained the reciprocal recombinant, which had the 5′ end of clone 32 and the 3′ end of clone 70. When the recombinants were constructed

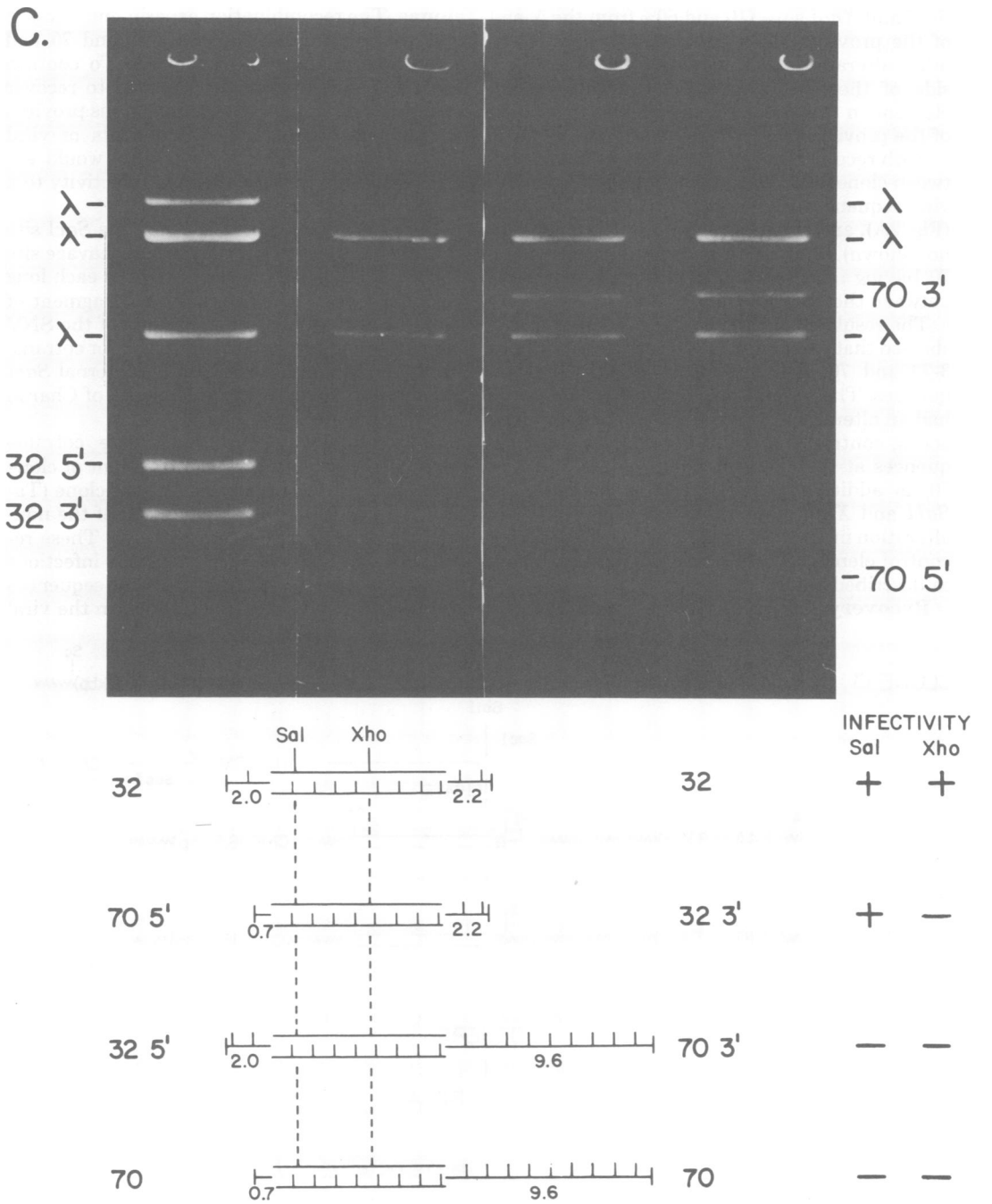


FIG. 2—*Continued*

with *Sal*I, the recombinant with the 3′ end of clone 70 was not infectious, and the reciprocal recombinant was. These results map the site affecting infectivity in clone 70 in the viral sequences to the 3′ side of the *Sal*I site or in the cellular sequences at the 3′ end of the provirus.

When *Xho*I was used to construct recombinants between clones 32 and 70, the recombinant with the 5′ end of clone 70 and the 3′ end of clone 32 was not infectious, nor was the reciprocal recombinant. These results showed that clone 70 had an alteration in the viral sequences between the

*Sal*I and *Xho*I sites (10 and 60% from the 5′ end of the provirus), and, in addition, there is another alteration in the viral sequences to the 3′ side of the *Xho*I site or a *cis*-acting control element in the cellular sequences at the 3′ end of the provirus.

Both recombinants constructed with *Xho*I between clone 3-73, containing an alteration in the viral sequences between the *Sal*I and *Xho*I sites (Fig. 2A), and clone 70 were not infectious (data not shown). This result is consistent with clone 70 having an alteration in the viral sequences between the *Sal*I and *Xho*I sites.

The results of the recombination experiments showed that two noninfectious proviruses, clones 3-73 and 70, had alterations in the viral sequences. The noninfectious provirus of clone 63 had an alteration in the viral sequences or a *cis*-acting control element in the cellular DNA sequences at the 3′ end of the provirus. In clone 70, in addition to the alteration between the *Sal*I and *Xho*I sites, there was either another alteration in the viral sequences or a 3′ *cis*-acting control element. These possibilities were tested as described below.

Recovery of virus from noninfectious clones. The recombination experiments showed that the proviruses in clones 3-73 and 70 had alterations in the viral sequences. To confirm further these experiments, we tried to recover virus by transfection of a noninfectious provirus in the presence of cloned fragments of viral DNA. This technique, if successful, would enable us to map the site affecting infectivity to a particular fragment of viral DNA.

The provirus of clone 13 lacked the *Sac*I site at 0.76 kbp (Fig. 1). The only other cleavage site for *Sac*I in SNV DNA was the site in each long terminal repeat. The internal *Sac*I fragment of clone 13, therefore, contained most of the SNV sequences and was potentially useful for cotransfections. We therefore cloned the internal *Sac*I fragment of clone 13 in the *Sac*I site of Charon 10 (29) as described in Fig. 3.

Chicken embryo fibroblasts were cotransfected pairwise with the *Sac*I fragment of clone 13 and DNA from each noninfectious clone (Table 1). Virus was recovered with all of the noninfectious proviruses except clone 13. These results showed that clone 63 was not infectious because of an alteration in the viral sequences and that clone 70 had an alteration in the viral

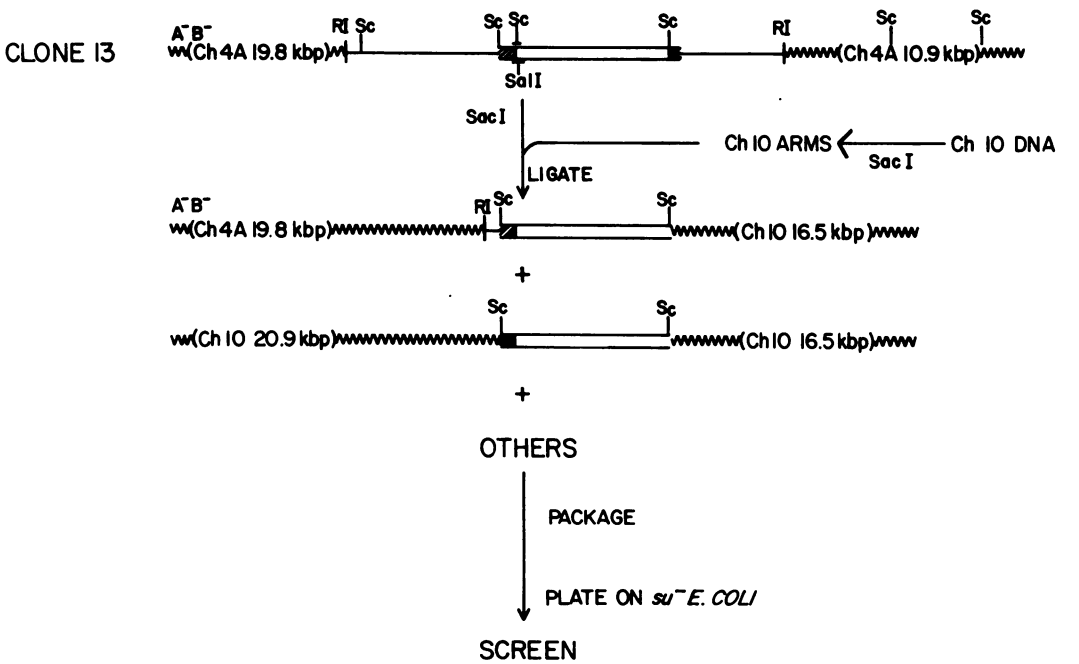


FIG. 3. *Subcloning of the internal SacI fragment from clone 13. One-half microgram of SacI-digested clone 13 DNA was mixed with a fivefold excess of SacI arms of Charon 10 (Ch10). DNAs were ligated (22) and packaged in vitro (5). The resultant phage were plated on an Su⁻ E. coli strain and screened with nick-translated SNV [^{32}P]DNA according to the technique of Benton and Davis (3). Sawtooth lines indicate lambda sequences, single lines indicate cellular sequences, and double lines indicate viral sequences. The hatched box represents the long terminal repeat in SNV DNA. T indicates the absence of a restriction enzyme cleavage site in the viral DNA.*

sequences to the 3′ side of the *Xho*I site rather than a *cis*-acting control element in the cellular sequences at the 3′ end of the provirus. (This alteration was in addition to the alteration between the *Sal*I and *Xho*I cleavage sites; Fig. 2C).

TABLE 1. *Recovery of virus after cotransfection of noninfectious proviruses with the SacI fragment of clone 13*[a]

Clone	Infectivity
13	−
3-73	+
63	+
70	+

[a] Dilutions of DNA of the indicated noninfectious clones were coprecipitated with 10 μg of salmon sperm DNA and 10 μg of *Sac*I-digested DNA from Charon 10 *Sac*I clone 13 per ml of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline solution. The precipitate (0.2 ml) was used to transfect 3×10^5 chicken embryo fibroblasts on a 35-mm plate. +, $>10^2$ IU per μg of noninfectious clone DNA; −, <1 IU per μg of noninfectious clone DNA.

In addition, this result confirmed that clone 3-73 had an alteration in the viral sequences.

The *Bgl*II fragments of clone 32 which contained viral DNA were subcloned in pHUB4 to use in cotransfections (described in the legend to Fig. 4). These fragments were used to map the site affecting infectivity of clone 13 and to map roughly the location of the alterations in the other noninfectious proviruses. Figure 4 shows a map of these *Bgl*II fragments and the results of the cotransfection experiments. Virus was recovered from clones 13, 3-73, and 63 after transfection with the same *Bgl*II fragment of clone 32. This result indicated that the provirus of clone 13 had an alteration in the 5′ viral sequences which made the provirus noninfectious or that the 5′ cellular sequences influenced expression. The alterations in the proviruses of clones 3-73 and 63 mapped in the 5′ viral sequences between the *Sal*I site at 0.86 kbp and the *Bgl*II site at 1.8 kbp (these results and Fig. 2).

The provirus of clone 70 did not produce

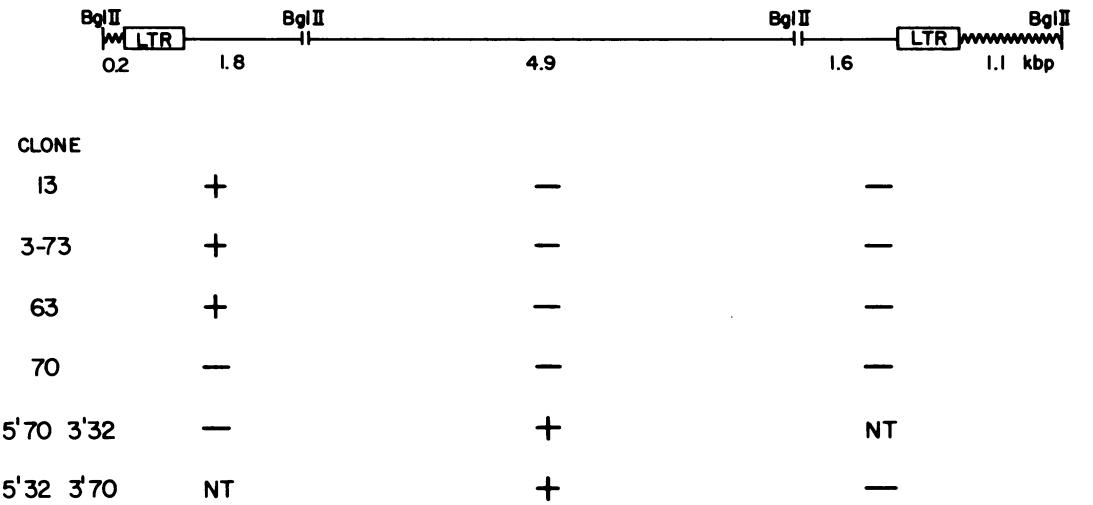


FIG. 4. *Cotransfection of noninfectious clones and BglII fragments of clone 32. Fragments of clone 32 containing SNV DNA were subcloned in the BglII site of pHUB4 (4). One-half microgram of phosphatase-treated, BglII-digested pHUB4 DNA was mixed with 0.1 μg of BglII-digested clone 32 DNA in 100 μl of DNA ligase buffer (22). One-tenth unit of DNA ligase from T4-infected E. coli cells was added. Ligation was carried out at 12°C for 12 to 16 h. The DNA was then used to transform Ca^{2+}-shocked HB101 cells (28). Transformed cells were selected on LB plates containing kanamycin (25 μg/ml). Colonies which contained SNV DNA were detected by the procedure of Grunstein and Hogness (12), using nick-translated SNV [^{32}P]DNA. To screen the plasmids, DNA from minipreps (prepared as described in Materials and Methods) was digested with BglII, and the fragments were separated on agarose gels. Bulk preparation of recombinant plasmid DNA was done according to published procedures (6, 14). Dilutions of DNA of the four noninfectious clones were coprecipitated with 10 μg of salmon sperm DNA and approximately 10 μg of BglII-digested DNA of each of these plasmids per ml of HEPES-buffered saline solution (8). The precipitates (0.2 ml) were used to transfect 3×10^5 chicken embryo fibroblasts on a 35-mm plate. Symbols: (+) $>10^2$ IU per μg of noninfectious clone DNA; (−) indicates <1 IU per μg of noninfectious clone DNA. Map shows BglII fragments of clone 32 containing SNV DNA. Sawtooth lines indicate cellular sequences, and smooth lines indicate viral sequences. The box indicates the long terminal repeat (LTR).*

infectious virus after transfection with the *Bgl*II fragments of clone 32. The alteration in clone 70 was not at a *Bgl*II cleavage site, since clone 70 had both of these. These results are consistent with the mapping by construction of recombinants, which indicated that clone 70 had a double alteration (see above). One of these alterations was mapped above between the *Sal*II and *Xho*I cleavage sites at 0.86 and 4.9 kbp, respectively. The other was in the viral sequences to the 3′ side of the *Xho*I site.

To map the alterations in the provirus of clone 70, the recombinants between the provirus of clone 70 and the provirus of clone 32, constructed with *Xho*I (see Fig. 2C), were used in cotransfections with the *Bgl*II fragments of clone 32. For both recombinant proviruses, virus was recovered by transfection with the 4.9-kbp internal fragment of clone 32 (Fig. 4). This result, along with those of Fig. 2C, indicated that the alterations in clone 70 mapped between the *Bgl*II site at 1.8 kbp and the *Xho*I site at 4.9 kbp and between the *Xho*I site and the *Bgl*II site at 6.7 kbp. The failure to recover virus in cotransfections of clone 70 and this fragment is unexplained. Possibly, one or both alterations were near the *Bgl*II cleavage sites, causing a reduced efficiency.

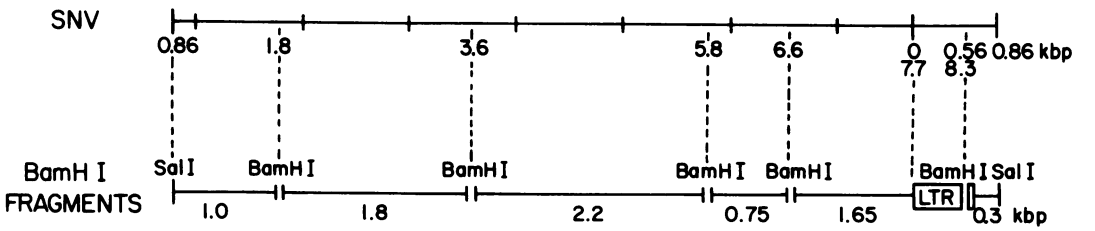
The cotransfection of clone 13 with the *Bgl*II fragments of clone 32 did not eliminate the possibility that the cellular sequences at the 5′ end influenced expression. To determine whether clone 13 had an alteration in the viral DNA and to confirm the mapping of the alterations in the proviruses of clones 3-73, 70, and 63, we subcloned the *Bam*HI fragments of the 7.7-kbp *Sal*I fragment of clone 60B in pBR322. Clone 60B contains a tandem duplication of viral DNA (19). Since *Sal*I cleaves viral DNA once, digestion of DNA from clone 60B with *Sal*I generated a circular permutation of the viral DNA, 7.7 kbp in size. This fragment contained a complete copy of the viral sequences because, after subcloning in pBR322, the fragment was infectious if removed from the plasmid by digestion with *Sal*I (data not shown). The subcloning of the *Bam*HI fragments of clone 60B is described in the legend to Fig. 5, which shows a map of the *Bam*HI cleavage sites and the results of cotranfection of chicken embryo fibroblasts with DNA from the noninfectious clones and DNA from the plasmids containing the *Bam*HI fragments of viral DNA. The alterations of clones 13, 3-73, and 63 mapped in the same 1.0-kbp region between the *Sal*I site at 0.86 kbp and the *Bam*HI site at 1.8 kbp (these results and Fig. 2). The alteration affecting infectivity of clone 13 was not at the position of the lost *Sal*I cleavage site, since recovered virus DNA lacked this cleavage site (data not shown). Virus was not recovered from clone 70. This result is not surprising in light of the results indicating that clone 70 had a double alteration. The alterations in the recombinants between the proviruses of clones 70 and 32 mapped between the *Bam*HI cleavage sites at 1.8 and 3.6 kbp and between the *Xho*I cleavage site at 4.9 kbp and the *Bam*HI cleavage site at 5.8 kbp (these results and Fig. 2).

Pairwise transfections of noninfectious proviruses. The mapping by cotransfection with fragments of viral DNA could not resolve the positions of the alterations in the proviruses of clones 13, 3-73, and 63. These proviruses contained an alteration between the *Sal*I (0.86 kbp) and *Bam*HI (1.8 kbp) cleavage sites. If these alterations are at different locations in the viral sequences, then cotransfection of two noninfectious proviruses might produce virus. Pairwise combinations of all four noninfectious proviral DNAs were assayed for infectivity (Table 2). All combinations of two noninfectious proviruses were positive for the production of virus. These results indicated that the alterations in the proviruses of clones 13, 3-73, 63, and 70 are not at the same site. Figure 6 shows the position of these alterations.

DISCUSSION

We have mapped the sites affecting infectivity of four noninfectious proviruses of SNV cloned in the *Eco*RI site of Charon 4A. For all of the proviruses studied, the sites affecting infectivity are in the viral sequences. Virus was rescued from three of the four noninfectious proviruses by the same restriction endonuclease-generated fragments of viral DNA. However, pairwise transfections of these noninfectious proviruses showed that the alterations are not at the same site. These data indicate that the difference in infectivity of these proviruses from chronically infected cells is a result of alterations in the noninfectious proviruses rather than the effects of a *cis*-acting element in the adjacent cellular sequences of infectious or noninfectious proviruses.

This result leaves unanswered the question of why there is a correlation of infectivity with the size of the fragments generated with restriction endonuclease *Eco*RI which contain viral DNA (2, 16, 19). Possibilities which account for the finding are: cells with infectious proviruses at a limited number of sites could be selected during the acute phase; cellular mechanisms may exist which alter some proviruses; or cleavage sites for the restriction endonuclease *Eco*RI could be generated or lost by modifications, insertions, deletions, or rearrangements in the cellular sequences adjacent to some infectious proviruses.



CLONE						
13	+	−	−	−	−	−
3-73	+	−	−	−	−	−
63	+	−	−	−	−	−
70	−	−	−	−	−	−
5'70 3'32	−	+	−	NT	NT	NT
5'32 3'70	NT	NT	+	−	−	−

FIG. 5. *Transfection of noninfectious clones with the BamHI or BamHI-SalI fragments of the 7.7-kbp SalI fragment of clone 60B. The 7.7-kbp SalI fragment of clone 60B was cloned into the SalI site of pBR322, pBR60BS1, as described in the legend to Fig. 4 except that transformed cells were selected on ampicillin plates. The BamHI and BamHI-SalI fragments of the 7.7-kbp SalI fragment (shown in the map above the table as a SalI permutation of SNV DNA) were subcloned into the BamHI site or the BamHI-SalI sites of pBR322. Before digesting with BamHI, pBR60BS1 was digested with EcoRI and treated with alkaline phosphatase to prevent it from transforming cells. The 5' SalI-BamHI fragment was obtained by ligation of BamHI-digested pBR60BS1 at 10 μg of DNA per ml. The 3' BamHI-SalI fragment was cloned by using BamHI, since there is a BamHI site in pBR322 approximately 300 base pairs from the SalI site. To verify that recombinant plasmids contained only one viral DNA fragment, plasmid DNA preparations were analyzed with restriction endonucleases (data not shown). After digestion with the appropriate enzyme(s) (BamHI or BamHI and SalI), plasmid DNA was used at a concentration of 10 μg/ml in cotransfections as described in the legend to Fig. 4. Symbols: (+) $>10^2$ IU per μg of noninfectious clone DNA; (−) <1 IU per μg of noninfectious clone DNA. The scale above the BamHI fragments of the 7.7-kbp fragment of clone 60B indicates the position of the BamHI cleavage sites in unintegrated linear SNV DNA. LTR, Long terminal repeat.*

We have not determined the mechanism of virus production after cotransfection. The mechanism is probably different from the mechanism of marker rescue in simian virus 40, polyoma virus, and phage φX174 (18). The formation of heteroduplexes increases the efficiency of marker rescue of these viruses. Denaturation and annealing of SNV DNA did not increase the efficiency of recovery (data not shown).

Recombination apparently occurs in marker rescue of herpesviruses (20, 25), adenoviruses (1, 10), and avian leukosis-sarcoma viruses (7). The recovery of virus studied here probably involves recombination rather than complementation, since virus of greater than 10^6 PFU/ml was produced (data not shown). Furthermore, virus recovered from transfection of clone 13 with the *Sal*I-*Bam*HI fragment of viral DNA produced viral DNA which lacked the *Sal*I cleavage site (data not shown). Complementation would require transcription and translation of this fragment, which seems unlikely.

Previously, we described the isolation of 10 recombinant DNA clones containing SNV proviruses (19). Several of these proviruses have alterations in the viral DNA detectable by biochemical techniques. The proviruses of clones 13 and 32 have lost at least two cleavage sites (19), and the provirus of clone 44 has lost at least one (K. Shimotohno, personal communi-

TABLE 2. *Cotransfection with DNAs of noninfectious clones*[a]

Clone	Clone			
	13	3-73	63	70
13	−	+	+	+
3-73		−	+	+
63			−	+
70				−

[a] DNAs from two noninfectious clones were mixed, and the mixture was assayed for infectivity with salmon sperm DNA (10 μg/ml) as carrier (8). A plus indicates greater than 15 IU/μg. (Dilutions of DNA to 0.1 μg of each clone per plate [three plates] were positive for virus production.) A minus indicates less than 0.2 IU/μg. (0.5 μg of DNA of a noninfectious clone did not produce virus.)

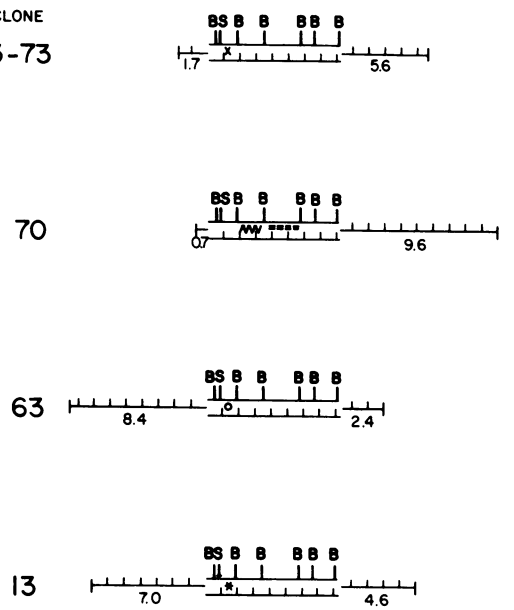


FIG. 6. *Positions of the alterations responsible for the lack of infectivity of the noninfectious proviruses. The alterations causing the lack of infectivity of the four noninfectious proviruses (×, sawtooth line, a broken double line, ○, and *) map between the adjacent restriction endonuclease cleavage sites. Cleavage sites in the viral DNA for SalI and BamHI are indicated by S and B, respectively. Vertical bars are separated by 1 kbp, and the numbers under the adjacent cellular sequences indicate their size in kilobase pairs. An upside down T indicates that the SalI site in clone 13 is missing.*

cation). The loss of the cleavage sites in clones 13 and 32 is not the result of a deletion spanning the two sites (data not shown; 19). All four noninfectious proviruses have alterations in the viral DNA which makes them noninfectious.

It is unlikely that these alterations arose during the molecular cloning. The isolation of several recombinant phage containing infectious proviruses (19) indicated that the initial cloning events did not alter the proviruses. These proviral DNAs have remained infectious after many generations in *Escherichia coli*. In addition, the size of the inserted *Eco*RI fragments containing the infectious proviruses and the size of the inserted *Eco*RI fragments containing noninfectious proviruses agree with the results of previous studies using DNA from mass populations of infected cells (15, 16). Finally, the loss of the *Sac*I cleavage site at 0.75 kbp has also been observed in DNAs from mass populations of infected cells (J. J. O'Rear, unpublished data) and in cell clones of SNV-infected rat cells (17).

These results, therefore, indicate that a high frequency of alterations occurs in the viral genome. These alterations could arise during cellular DNA replication, transcription of the provirus, or synthesis of viral DNA.

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