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Analysis by Microinjection of the Biological Effects of Site-Directed Mutagenesis in Cloned Avian Leukosis Viral DNAs

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Cloned avian leukosis viral DNAs were mutagenized in the long terminal repeat, in the leader sequence for *env* mRNA, and at the *pol-env* junction. The effect of these mutations in the viral DNA upon its ability to direct virus production or *env* mRNA synthesis was analyzed by microinjecting the mutant DNAs into chicken embryo fibroblasts and into chicken cells transformed by the *env*-deficient Bryan strain of Rous sarcoma virus, respectively. The results indicated that: (i) addition of up to 8 base pairs 19 nucleotides upstream of the Hogness box did not block transcription; (ii) deletion of 26 base pairs, including the tRNA primer binding site, allowed synthesis of all viral products and participation in recombination, but replication was blocked; (iii) deletion of fewer than 50 base pairs 250 bases downstream of the long terminal repeat depressed expression of all viral genes; and (iv) deletion of most of the *gag* and *pol* genes did not inhibit *env* mRNA synthesis, but virion packaging of the unspliced transcript was inefficient.

Genetic analysis and recombinant DNA technology permit studies of the biology and structure of avian retroviruses. With the technique of microinjection, it is possible to relate molecular structure directly to biological function. The objective of this work was to correlate the expression of several individual biological activities of the avian leukosis virus with defined regions of the cloned viral genome.

In recent studies, it was shown that cloned retroviral DNA injected into chicken cells is transcribed efficiently beginning soon after injection (11). Injections of intact viral genomic DNA into uninfected chicken embryo fibroblasts (CEF) result in the production of infectious virus, indicating that the injected DNA has directed synthesis of all essential viral molecules. When DNA is injected into cells transformed by the envelope glycoprotein-deficient Bryan strain of Rous sarcoma virus [RSV(−) cells], it is possible to sensitively assay the synthesis of the viral *env* mRNA alone. The transcription of injected DNA to produce functional nuclear precursor for *env* mRNA complements the RSV(−) deficiency and allows the release of focus-forming units (FFU) from injected cells (17).

In the work reported here, several genomic mutations were introduced into defined locations within the cloned viral genome by recombinant DNA technology. Microinjection was then used to determine the effect of these alterations upon the ability of the mutant DNA to participate in the production of *env* mRNA within injected RSV(−) cells or in the production of intact virus from CEF. The regions of the viral genome altered in these studies were the long terminal repeat (LTR), which is thought to contain the promoter and terminator for viral transcription; the first 300 to 400 transcribed bases, which form the leader sequence for *env* mRNA; and the junction of the *pol* and *env* genes, which contains the splice receptor site for *env* mRNA. Mutations were generated by removing nucleotides, except in the LTR, where nucleotides were added. The biological functions assayed included transcription of viral DNA, processing of RNA, expression of the *gag* and *pol* gene products, recombination, packaging of RNA into virions, and virus replication.

MATERIALS AND METHODS

Recombinant plasmids. Clone pLD12 was obtained from a leukosis viral recombinant between Rous-associated virus-2 *env* mRNA and RSV (19). pLD6 is a transforming clone of a Schmidt-Ruppin strain of RSV (manuscript in preparation). Recombinant phage λ clones containing a transformation-deficient Schmidt-Ruppin RSV were kindly provided by G. Ju and A. M. Skalka (8) and subcloned into pBR322. pL39td-2.4 has one copy of the LTR, and pL13td-2.2 has two copies of the LTR.

Site-specific mutagenesis. (i) The avian leukosis viral DNA contains several *Eco*RI restriction sites. Mutations were introduced into several of them after partial digestion of pL39td-2.4 DNA. Unit-length linear molecules were then separated on a 0.75% agarose gel containing 1 μg of ethidium bromide per ml, isolated by electroelution, and purified on DEAE-52 columns (20). The 5′ overhangs at the cleavage sites were filled

in by using the Klenow fragment of DNA polymerase I (3). The DNA was then ligated at 4°C overnight and used to transform *Escherichia coli* RRI (13). Clones were analyzed by the Birnboim minilysate procedure (2). Clones pL39R10, pL39R11, and pL39R15 were obtained with this procedure.

(ii) *Sac*I site mutations were produced after complete cleavage of pLD12 DNA at the single *Sac*I site. This DNA was then incubated with S1 nuclease at 14°C for various time periods (3). The protocol described above was used for cloning. Clones pL12S7 and pL12S19 were obtained with this procedure.

(iii) *Kpn*I site mutations were produced after cleavage of pL39td-2.4 DNA at the single *Kpn*I site. S1 nuclease treatment and cloning as described above led to the mutant plasmids pL39K2, pL39K11, and pL39K12.

(iv) pL13Bs2 was constructed by treating pL13td-2.2 DNA with restriction endonuclease *Bgl*II, religating the fragments, and then selecting for plasmids that had lost the internal 2.6-kilobase (kb) *Bgl*II fragment. The remaining *Bst*EII site was removed by S1 nuclease treatment as described above. The 2.6-kb *Bgl*II fragment was purified on an agarose gel and religated to the altered plasmid to reconstitute a complete viral genome. The correct orientation of the fragment was verified by restriction endonuclease analysis.

Mung bean nuclease treatment of pLD12 DNA which had been cleaved with *Sac*I was performed as follows. Mung bean nuclease was diluted with S1 buffer (3) to a concentration of 0.6 U/μl. A total of 2.4 U of enzyme per μg of DNA was added in a total volume of 20 μl, and the reaction mixture was incubated for various time periods at 37°C. The reaction was stopped by adding S1 stop solution (3) and extracted with 1 volume of phenol and 0.5 volume of chloroform. The protocol described above for *Eco*RI sites was then followed.

All plasmids were amplified, and the introduced mutations were confirmed in the purified DNAs by digestion with appropriate restriction endonucleases followed by electrophoresis on minigels (10).

DNA sequencing. The protocol of Maxam and Gilbert (12) was used.

Cell tissue culture. CEF preparations and culture conditions have been described previously (6). RSV(−) cells had been transformed by the use of UV-inactivated Sendai virus (9).

Microinjection. Injections and focus assays were performed as described (16, 17). Before injection, the viral DNAs were freed from vector by treatment with restriction endonuclease *Sal*I.

RESULTS

The restriction maps of the two retroviral DNA clones used in this work are shown in Fig. 1. Clone pL39td-2.4 contains three *Eco*RI sites within the virus-specific DNA and an additional site within the pBR322 part of the plasmid. One *Eco*RI site is located in the LTR just upstream of the Hogness sequence, which might be involved in the initiation of transcription (1, 4). To introduce a mutation at this site, the plasmid DNA was linearized by partial cleavage with restriction endonuclease *Eco*RI. Linear, full-length molecules were purified from agarose gels and treated with *E. coli* DNA polymerase I to fill in the single-stranded DNA at the cleavage site; this was followed by ligation and subcloning as described above. An analysis of the resulting mutants by treatment with *Eco*RI showed that one clone, pL39R10, had a more complex restriction pattern than expected (Fig. 2); therefore, this clone was further analyzed. Although pL39R10 was derived from a single, isolated colony, it seemed to contain a mixture of two different DNAs. Retransfection of *E. coli* RRI with purified pL39R10 DNA yielded two different clones, pL39R11 and pL39R15. An analysis of these DNAs showed that pL39R11 had lost the *Eco*RI site in the LTR, as well as that in the *gag* gene, whereas pL39R15 had lost only the *Eco*RI site in the LTR (Fig. 2). A possible explanation for the origin of these two plasmids is given below.

Both mutant plasmids were assayed biologically by microinjection into RSV(−) cells and uninfected CEF. Culture fluids from injected RSV(−) cultures were collected at 24-h intervals and tested directly for the titer of FFU, which would indicate the relative amount of active *env* mRNA transcribed from the injected DNA. Culture fluids from injected CEF cultures would contain only nontransforming leukosis viruses. These were assayed by determining their "helper" activity. The fluids from injected CEF cultures were used to infect RSV(−) cells, which would release FFU because of complementation with the *env* gene of the leukosis (helper) virus. The production of viral particles by injected CEF would require that the injected DNA had directed the synthesis of all viral functions needed for viral synthesis. Injections were normally performed with saturating DNA concentrations of 200 to 400 μg/ml. It has been shown previously that viral DNA concentrations above 20 μg/ml do not increase the number of viral particles produced (11).

DNA from clone pL39R15, which lacked only the *Eco*RI restriction site in the LTR, promoted virus production from CEF and FFU production from injected RSV(−) cells (Table 1), indicating that the introduction of up to 8 nucleotides at a position 19 bases (8) upstream of the Hogness sequence did not block transcription within a living host cell. Since saturating DNA concentrations were injected, a minor alteration in transcription efficiency might not have been detected in this analysis. Clone pL39R11, which lacked the *Eco*RI sites in both the LTR and the *gag* gene, directed FFU production from injected RSV(−) cells but not virus production from CEF (Table 1). The FFU production indicated that the DNA was transcribed, whereas the inability to direct virus production from CEF

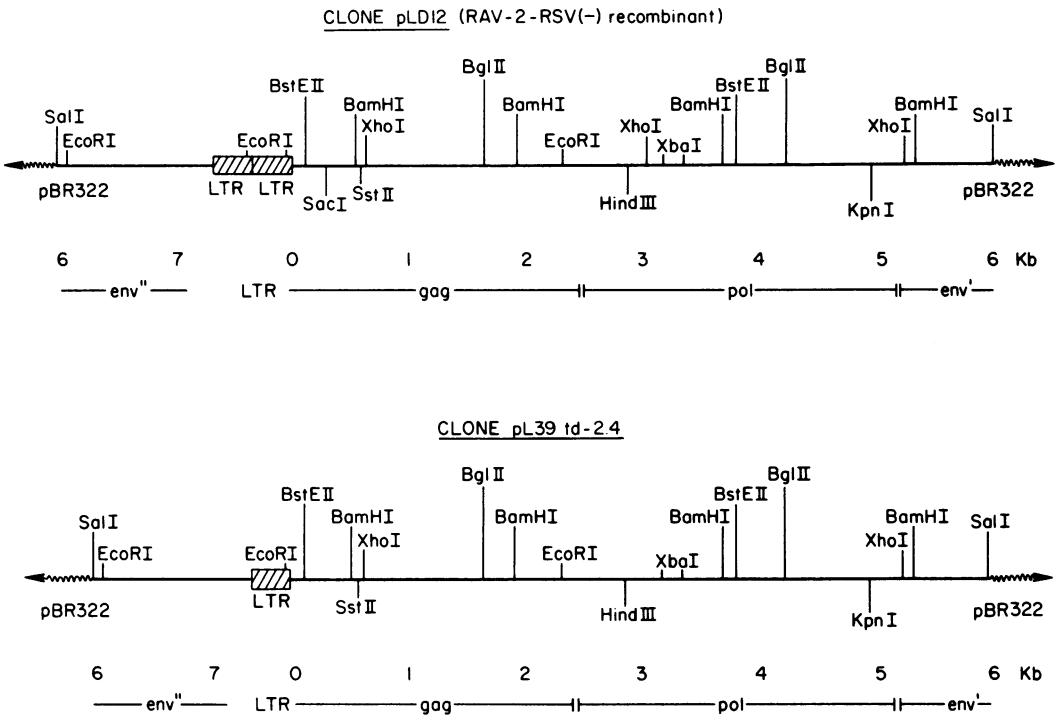

FIG. 1. Restriction maps of clones pLD12 and pL39td-2.4. A restriction endonuclease analysis of pLD12 and pL39td-2.4 revealed a close similarity to the restricton map of Prague C RSV DNA (D. Schwartz, R. Tiziard, and W. Gilbert, personal communication). Therefore, the numerical positions of the restriction sites shown are in accordance with the Prague C DNA sequence. Viral genes listed are the *gag* gene, the *pol* gene, and the *env* gene (which contains the *Sal*I cloning site and therefore is permuted in these clones).

indicated that at least one viral product, presumably from the altered *gag* gene, had not been produced. Coinjection of pL39R11 DNA with a polymerase-deficient mutant DNA is discussed below.

A second site of interest was the *Bst*EII restriction site at position 103, which coincides with the tRNA primer binding site for reverse transcriptase (7). The clones used contain a second *Bst*EII site at position 3,807 in the *pol* gene (Fig. 1). To remove this second site, pL13td-2.2 DNA was cleaved with *Bgl*II and religated, thereby removing a 2.6-kb region between the two *Bgl*II sites in which this second *Bst*EII site was located. The resulting deletion mutant, which contained only one *Bst*EII site at the primer binding site, was cleaved with restriction endonuclease *Bst*EII and treated with S1 nuclease as described above to remove nucleotides at and adjacent to the restriction site. After ligation and analysis, a clone lacking the *Bst*EII site was obtained. To reconstitute the viral genome, it was necessary to reintroduce the previously deleted *Bgl*II fragment into the altered plasmid in the correct orientation (see above). A DNA sequence analysis of the resulting clone, pL13Bs2, showed that in comparison to the sequence of Prague C (D. Schwartz, personal communication), nucleotides 101 to 126 had been removed by S1 nuclease (Fig. 3); thus, the entire primer binding site was deleted.

Microinjection of pL13Bs2 DNA into RSV(−) cells promoted FFU production, indicating that the DNA directed efficient *env* mRNA production. No helper virus was released from injected CEF (Table 2), possibly due to the inability of the full-length viral RNA transcript to direct the synthesis of active viral DNA. If only the replication function was altered in the pL13Bs2 mutant, the mutant would be expected to produce *gag* and *pol* gene products and to participate in recombination. To test for these possibilities, pL13Bs2 DNA was injected into cells transformed by a deletion mutant of a transforming Schmidt-Ruppin RSV DNA clone (pLD6) lacking part of both the *gag* and *pol* genes. FFU were produced after these injections (data not shown), indicating that the injected DNA supplied both the *gag* and *pol* functions. In addition, pL13Bs2 DNA was coinjected with DNA of the *gag*- and *pol*-deficient transforming mutant mentioned above and also, in a separate experiment,

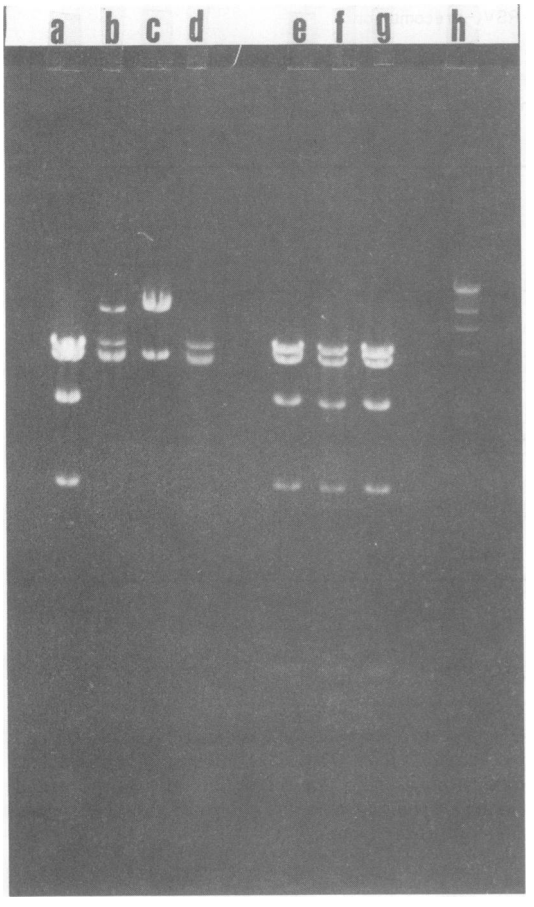

FIG. 2. Agarose gel analysis of mutant clones. Each DNA (1 μg) was digested with 1 U of *Eco*RI in a total volume of 10 μl for 60 min at 37°C. The fragments were then separated on a 1% agarose gel. Lane a, pL39td-2.4; lane b, pL39R10; lane c, pL39R11; lane d, pL39R15; lane e, pLD12; lane f, pL12S7; lane g, pL12S19; lane h, phage λ DNA size marker. Lanes c and d show that the corresponding DNAs are subsets of DNA shown in b.

with DNA of a larger deletion mutant of the same clone that lacked the *gag*, *pol*, and *env* genes. In both cases, FFU were released from the injected CEF, indicating that pL13Bs2 had produced all three viral gene products necessary for virus production (Table 2). Virus released from injected CEF in these experiments was able to transform infected cells because transcripts of the deleted transforming viral DNA had been packaged into infectious virions. Because of the deletion, this transforming virus would be defective in subsequent replication. Replication-competent transforming virus was also recovered from injected cultures, however, indicating that the pL13Bs2 DNA had participated in recombination with the deleted transforming virus (Table 2). As predicted, the only apparent defect in pL13Bs2 was the inability to participate in replication.

As described above, leukosis viral clone pLD12 contains a single *Sac*I site at position 255 (Fig. 1), which is between the primer binding site and the putative splice donor site for *env* mRNA (5). To determine the effect of a mutation in this region on *env* mRNA synthesis, pLD12 DNA was linearized with restriction endonuclease *Sac*I, treated with either S1 nuclease or mung bean nuclease before ligation, and recloned as described above. Only deletion mutants that lacked the *Sac*I site and were similar in size to the parental clone pLD12 were further analyzed (Fig. 2).

Of the clones derived by S1 nuclease treatment, pL12S7 and pL12S19 were further studied. Clone pL12S19 produced infectious virus after being injected into CEF (Table 1). It functioned approximately 30% as efficiently in the *env* complementation assay as did the parental clone pLD12. pL12S7 DNA, on the other hand, produced no virus after injection into CEF but functioned approximately 10% as efficiently as did pL12S19 in the *env* complementation assay (Table 1 and unpublished data). DNA sequence studies in the region of the deletion showed that a sequence of 11 bases in pL12S19 was replaced

TABLE 1. Biological activities of mutant clones lacking either *Eco*RI restriction sites or *Sac*I restriction sites[a]

Cells injected	FFU titer with DNA injected from:				
	Clones lacking *Eco*RI restriction sites		*Sac*I mutants and parental clone		
	pL39R11	pL39R15	pL12S7[b]	pL12S19	pLD12
RSV(−) cells					
1[c]	0	4	1	80	192
2	4	57	25	1,848	2,338
3	34	400	209	5,000	5,000
CEF (helper virus)	−	+	−	+	+

[a] DNA from the clones listed was injected into RSV(−) cells, and culture fluids were collected for 3 days and assayed for titer of FFU. The same DNAs were also injected into CEF. Culture fluids collected 3 days after injection into CEF were used to infect RSV(−) cultures from which culture fluids were collected after 3 additional days and tested for the presence of FFU. The production of FFU from infected RSV(−) cultures indicated the presence of helper virus released from injected CEF. Variations in FFU titer between experiments are due to variations in recipient cells.

[b] Titer of FFU was determined from a different but comparable experiment.

[c] Day after injection into RSV(−) cells.

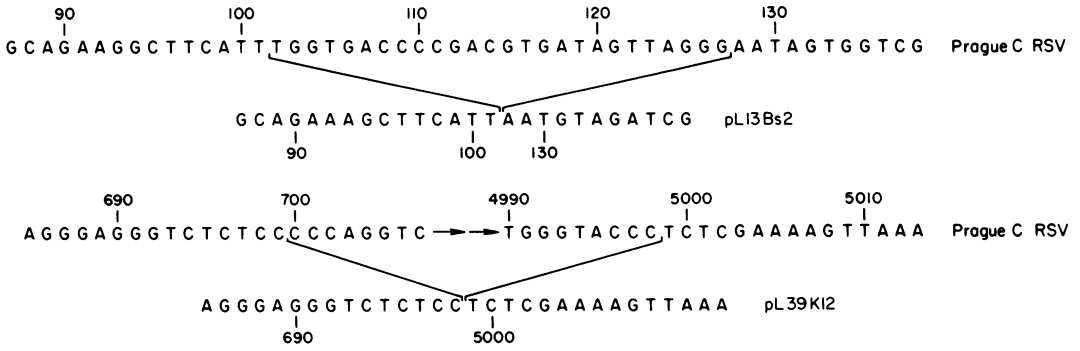

FIG. 3. Comparison of the DNA sequences flanking the deletions in clones pL13Bs2 and pL39K12 with the corresponding sequences in Prague C RSV DNA (D. Schwartz, R. Tiziard, and W. Gilbert, personal communication). Arrows indicate the sequence from position 701 to 4,989. Base changes are found between the two RSV strains.

by a sequence of 4 bases in pL12S7 (data not shown). If this sequence difference accounts for the different biological activities of pL12S7 and pL12S19, then this region of the genome undoubtedly has an important yet unknown biological function. This conclusion, however, must await further studies to eliminate the possibility that a second mutation in pL12S7 at an unknown site might account for its depressed biological function.

Eight more mutants whose *Sac*I sites were deleted by mung bean nuclease treatment exhibited a wide variety of activities after injection into RSV(−) cells. The significance of these variations is under investigation.

env mRNA is generated by the splicing of a 5′ leader sequence to the *env* gene (21). The splice receptor site is postulated to be located near the *pol-env* junction (D. Schwartz, R. Tiziard, and W. Gilbert, personal communication) in a region near the unique *Kpn*I restriction site in the retroviral clones that we used (Fig. 1). pL39td-2.4 DNA was linearized by cleavage with the restriction endonuclease *Kpn*I and treated with S1 nuclease as described above. The resulting clones which had lost the *Kpn*I site were further studied. Two clones, pL39K2 and pL39K11, had lost fewer than 100 bases according to agarose gel analysis, whereas clone pL39K12 had most of the *gag* and *pol* genes deleted.

When pL39K2 and pL39K11 were injected into RSV(−) cells, FFU were released, indicating that the *env* gene was intact and that normal splicing of *env* mRNA had occurred (Table 3). No virus was detected after CEF injections with either plasmid, suggesting that the bases deleted near the *Kpn*I site were required for *pol* activity. The pL39R11 mutant, previously assumed to be deficient only in the *gag* gene, was coinjected with pL39K11 into CEF to determine whether the two DNAs might complement each other, leading to helper virus production. In only one of four experiments was virus detected after such injections (data not shown), suggesting that

TABLE 2. Biological activity of mutant clone pL13Bs2[a]

Cells injected	FFU titer 1 day after DNA injected from:				
	pL13Bs2	pLD12	pL13Bs2 + pL6BH7[b] (*src*$^+$)	pL13Bs2 + pL6Bg19/9[c] (*env*$^+$ *src*$^+$)	pLD12 + pL6Bg19/9[c]
RSV(−) CEF	140	312	20	156	51
Helper virus	−	+	+	+	+

[a] DNA from the clones listed was injected into RSV(−) cells and CEF and analyzed for titer of FFU and production of helper virus as described in Table 1. pL13Bs2 was also coinjected into CEF with an equivalent amount of DNA from deletion mutants of transforming viral clone pLD6. Culture fluids were collected from injected CEF and tested directly for FFU, the presence of which indicated that the coinjected DNAs had complemented each other. To test for recombinants, culture fluids were collected from focus assay plates and tested for presence of transforming virus. Production of FFU by cells infected with culture fluids from injected CEF indicated that recombination had occurred. pLD12 is a lymphoid leukosis viral clone.

[b] Clone pL6BH7 was derived from a clone of Schmidt-Ruppin B and had 4.8 kb deleted between the *Bam*HI restriction sites in the *gag* and *env* genes, retaining only a functional *src* gene (manuscript in preparation).

[c] Clone pL6Bg19/9 was derived from a clone of Schmidt-Ruppin B and had 2.6 kb deleted between the *Bgl*II restriction sites in the *gag* and *pol* genes; it therefore had functional *env* and *src* genes.

TABLE 3. Biological activities of mutant clones with deletions near the *pol-env* junction[a]

Day after injection	FFU titer with DNA injected from				
	pL39K2	pL39K11	pL39K12	pL39X1	pL39X2
1	16	15	330	0	0
2	552	504	496	0	0
3	1,824	2,000	648	0	0

[a] DNA from the clones listed was injected into RSV(−) cells. Culture fluids were collected daily and assayed for titer of FFU. Clones pL39X1 and pL39X2 had all 4,625 base pairs between the *Xho*I restriction sites deleted. The slow increase in FFU titer in cultures injected with pL39K12 suggests that helper virus production was diminished compared with that in cultures injected with pL39K2 or pL39K11.

complementation or recombination was inefficient. The infrequent complementation might have been due to the inefficient transcription of coinjected DNAs, although virus was actively produced when pL13Bs2 was coinjected with deleted transforming virus clones. Alternatively, the mutation in the *gag* gene of pL39R11 might have depressed the activity of its *pol* gene enough to render it ineffective in complementing the *pol* mutation in pL39K11.

The deletion mutant pL39K12 directed the production of FFU from injected RSV(−) cells more actively than did pL39K2 or pL39K11 (Table 3), indicating that the entire *env* gene was retained and that splicing had occurred. A DNA sequence analysis showed that the deletion extended from nucleotide 699 to 4,987 (analogous to Prague C); therefore, the entire deletion was localized in the *env* mRNA intron (Fig. 3; D. Schwartz, R. Tiziard, and W. Gilbert, personal communication).

Although pL39K12 DNA was more active in the *env* complementation assay than were other *Kpn*I-site mutants, it appeared to direct the production of fewer *env*-containing helper virus particles from RSV(−) cells (Table 3). A careful analysis of the FFU-to-helper virus ratio after RSV(−) cell injections was therefore undertaken. The helper virus titer was estimated by an end-point dilution analysis on RSV(−) cultures. Compared with pL39K11 DNA, the deletion mutant pL39K12 produced eightfold-more infectious virus (Table 4). The FFU-to-helper virus ratio, however, was 40:1 for pL39K12 and 1:2.6 for pL39K11, confirming that the pL39K12 DNA directed the synthesis of fewer helper virus particles than did the other clones tested. The activity of pL39K11 was similar to that of the parental clone (data not shown). The possibility that either RNA processing or viral RNA packaging was responsible for the altered activity of pL39K12 is discussed below.

The deletion in pL39K12 eliminated all but 341 nucleotides between the two *Xho*I sites in clone pL39td-2.4 (Fig. 1). To determine whether sequences essential to *env* mRNA synthesis were included within these 341 bases, pL39td-2.4 DNA was cleaved with the restriction endonuclease *Xho*I and religated to generate a clone with a single *Xho*I site that lacked all sequences between the original sites. The DNAs of two clones, pL39X1 and pL39X2, were injected into RSV(−) cells, but neither directed the synthesis of any active *env* mRNA to promote FFU production (Table 3). It is likely that these clones were inactive because the splice receptor site for *env* mRNA had been deleted.

DISCUSSION

Cloned retroviral DNA is transcribed soon after microinjection into cultured chicken cells, before major alterations are likely to occur in the injected DNA (11). It is therefore possible to study the biological activity of mutagenized viral DNA directly by using microinjection coupled with viral genetics.

The region of single-stranded DNA produced during restriction endonuclease cleavage at the *Eco*RI site located in the LTR 19 bases upstream of the Hogness sequence would have been duplicated when DNA polymerase I was used. The DNA of the resulting clone, pL39R15, directed the synthesis of *env* mRNA as shown by FFU production from injected RSV(−) cells. Virus production from injected CEF cells indicated that pL39R15 DNA also directed all other viral activities necessary for virus production. Mutant clone pL39R11 lacked the *Eco*RI sites in the LTR as well as in the *gag* gene and directed active *env* mRNA production but not virus production from CEF (Table 1).

The fact that an alteration in the genome near the Hogness sequence did not block transcription is consistent with results from other in vivo

TABLE 4. FFU-to-helper virus ratio after injections of DNA from pL39K11 and pL39K12[a]

Clone	FFU titer	Helper virus titer	Ratio of FFU to helper virus
pL39K11	160	422	0.38
pL39K12	2,060	53	39

[a] DNA from clones pL39K11 and pL39K12 was injected into RSV(−) cells. After 22 h, culture fluids were collected and assayed for titer of FFU. RSV(−) cultures were then infected with twofold serial dilutions of culture fluids from injected cells. The infected cells were subcultured once, and after 3 days culture fluids were collected and assayed for the presence of FFU. The titer of helper virus listed indicates the highest dilutions of culture fluids from injected cells which contained helper virus.

studies (1, 4). It may not be possible from this type of study, however, to detect a subtle alteration in transcriptional efficiency, since injected DNA concentrations in this work were up to 10-fold higher than that required to cause maximal virus production (11). The saturating DNA concentration within injected cells might have overcome the effect of a decrease in the transcriptional efficiency of the injected DNA. The second mutation within the *gag* gene of pL39R11 at position 2,316 (Fig. 1) might also have had an effect upon *pol* gene activity, since pL39R11 was very inefficient in complementing a coinjected *pol* mutant (pL39K11).

Mutants pL39R11 and pL39R15 were obtained from the same isolated bacterial colony. The two plasmids might have been generated when the parental plasmid DNA was linearized at the *Eco*RI site in the LTR, and, by coincidence, the *Eco*RI site in the *gag* gene was nicked. The DNA polymerase I might then have repaired the *gag* gene nick incorrectly, whereas the single-stranded regions at the LTR cleavage site were duplicated. After transfection, the replication of the resulting molecule could then have led to two plasmid populations, one of which had lost two *Eco*RI restriction sites.

It was interesting that the elimination of 26 bases, including the primer binding site in clone pL13Bs2, did not alter the synthesis of any viral gene product or the ability of the virus to participate in recombination (Table 2). The inability of DNA from this clone to promote virus production from injected CEF was consistent with the prediction that the RNA transcript could not be replicated by reverse transcriptase. Other studies have shown that microinjected DNAs do not participate in recombination (unpublished data). It therefore appears likely that recombination occurs after the synthesis of viral RNA, presumably during reverse transcription.

Deletions around the *Sac*I site, which is located between the primer binding site and the splice donor site for *env* mRNA, led to decreased activity in *env* mRNA production. Clone pL12S7 exhibited little *env* mRNA activity and did not promote virus production from injected CEF (Table 1), whereas a closely related mutation, pL12S19, directed FFU production from RSV(−) cells and virus production from CEF at rates closer to that observed with the parental virus. Although we cannot yet exclude the possibility of a second mutation in pL12S7, these data indicate that the region around the *Sac*I site might be important for the synthesis of all viral gene products. The role of this region of the viral genome in virus replication is not known.

Of particular interest was mutant pL39K12, in which 4,292 base pairs had been removed by S1 nuclease treatment at the *Kpn*I restriction site (Fig. 1). Although most of the *gag* and *pol* genes had been removed from this clone, transcription and splicing occurred well, to produce *env* mRNA. Although *env* mRNA was produced more actively than with clones pL39K11 or pL39K12, which had smaller deletions introduced at the *Kpn*I site (near the *pol-env* gene junction), the full-length transcript of pL39K12 was packaged into virus much less efficiently than were the mutants with smaller deletions (Table 3).

To explain the difference in activity and relative helper virus production of pL39K12 compared with pL39K11 (Table 4) and the parental virus clone pL39td-2.4, the following possibilities may be considered. First, there might be a sequence or region in the viral genome, deleted in pL39K12, which is required for efficient packaging of the RNA transcript into viral particles. The buildup of an unpackaged, full-length pL39K11 transcript within the cytoplasm might then have resulted in the splicing of a greater proportion of newly transcribed pL39K12 to yield *env* mRNA, thus increasing the number of infectious viral particles released from the *env*-deficient RSV(−) cells. Alternatively, the smaller size of pL39K12 might account for a relatively greater number of transcripts. It is not expected that a full-length pL39K12 transcript would itself serve as *env* mRNA, because the translation product would contain 84 amino acids of *gag* proteins (Fig. 1) in front of the envelope glycoprotein signal sequence, which would probably inhibit proper glycosylation.

A second explanation for the activity of pL39K12 involves the delicate control of viral RNA splicing, which accounts for partial splicing of full genomic transcripts to yield a balance of *env* mRNA, genomic RNA, and larger viral mRNAs (18, 21). It is possible that the proper control of splicing requires a larger transcript or that in pL39K12 a control element for processing might have been removed, resulting in a greater proportion of pL39K12 transcript processed to *env* mRNA. This would explain a high proportion of *env* mRNA and relatively little full transcript being available for virus packaging to yield helper virus. There is evidence that *env* mRNA itself can be reverse transcribed to produce an active subgenomic provirus able to complement RSV(−) cells (15). It is therefore unlikely that the small size of the pL39K12 transcript could account for greatly reduced activity in provirus formation.

The 3′-terminal sequences of the *pol* gene may code for a biologically active molecule, an endonuclease (14). The deleted sequences in pL39K11 and pL39K2 might have been within the region coding for this endonuclease. At present we have no evidence, however, that

TABLE 5. Summary of clones analyzed

Clone	Site mutated	Genomic location affected	*env*	Helper virus
pLD12	None		+	+
pL39R11	*Eco*RI	LTR *gag* (*pol*)	+	−
pL39R15	*Eco*RI	LTR	+	+
pL13Bs2[a]	*Bst*EII	Primer binding site	+	−
pL12S7	*Sac*I	mRNA leader (2nd site)	±	−
pL12S19	*Sac*I	mRNA leader	+	+
pL39K2	*Kpn*I	*pol*	+	−
pL39K11	*Kpn*I	*pol*	+	−
pL39K12[b]	*Kpn*I	*gag pol*	++	−
pL39X1	*Xho*I	*gag pol env*	−	−
pL39X2	*Xho*I	*gag pol env*	−	−

[a] This clone had all structural genes and participated in recombination; it did not participate in replication.
[b] This clone had elevated *env* mRNA levels and depressed helper virus productivity.

these altered DNAs fail to direct virus production from CEF because of the absence of an endonuclease.

In addition to the deletion mutant pL39K12 studied in this work, other deletion mutants have been analyzed in separate studies (manuscript in preparation). As with pL39K12, deletions within the *env* mRNA intron did not block production of *env* mRNA after injections into RSV(−) cells. When either the splice donor site or the splice receptor site was deleted, however, *env* mRNA production was blocked.

Table 5 summarizes the work reported here. It is apparent that microinjection coupled with viral genetics and recombinant DNA technology provides a powerful approach to the correlation of polynucleotide structure and biological activity. With this approach it will be possible to analyze those genomic regions which participate in the varied biological activities of the virus.

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