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Differences in Intracellular DNA Ligation After Microinjection and Transfection

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An uninterrupted avian sarcoma viral genome terminated by viral long terminal repeat sequences was cloned into a pBR322 plasmid. After introduction into a cultured avian cell, transcription of either the circular plasmid molecule or one linearized within the pBR322 sequences could initiate and terminate at long terminal repeat sequences, yielding full-sized viral RNA. A plasmid DNA molecule linearized by cleavage within the viral *pol* gene, on the other hand, would have to undergo ligation to yield full-sized viral RNA. Microinjection of each of these three types of DNA into the nuclei of quail cells promoted the release of similar virus titers, indicating that the plasmid DNA cleaved within the viral *pol* gene had been efficiently and accurately ligated. When plasmid DNA was transfected into quail cells, circular and pBR322-cleaved molecules directed the synthesis of similar virus titers, indicating that they were similarly taken up and utilized by the cells. Compared with these results, plasmid DNA cleaved within the *pol* gene was reduced in activity over 95% after transfection. This reduction did not result from inefficient ligation but from the generation of mutations (of limited size) during ligation of the transfected molecules. Mutations were not observed after microinjection even into the cytoplasm. Consistent with these findings, transfected DNA termini were found to be joined regardless of their structure, whereas ligation after microinjection required that single-stranded protruding DNA termini be complementary.

For many biological studies, it has been valuable to introduce cloned DNAs into cells cultured from higher organisms where a wide range of biological activities may be examined against the background of cellular regulation. Physical (5, 8, 29) as well as biological (9, 18, 30) techniques have proven effective in placing purified macromolecules into cultured cells. In this study, we demonstrate that the immediate fate of DNAs within recipient cells depends upon the technique used for their introduction.

Transfection is used commonly to introduce DNA into cultured cells. In this technique, the purified DNA is placed in contact with cells in the form of a calcium phosphate precipitate (9) or in the presence of DEAE-dextran (18, 32). The DNA is believed to enter the cell by endocytosis and escape free into the cytoplasm after rupture of lysosomes (17). This process requires up to 24 h before transcription of input DNA is observed and commonly involves partial degradation of molecular termini (16, 26, 34). Once within the nucleus the input DNA molecules appear to be joined together (21, 23).

Microinjection has also been used to introduce foreign DNA into living cells (1, 3, 14). Although fewer cells can be treated with this technique, the amount of DNA introduced per cell can be controlled, carrier DNA is unnecessary, most cultured cells can be treated, and recipient cells are identifiable. Of significance is the fact that with microinjection the adverse effects upon the cell are minimal. For example, cloned retroviral DNA is not only transcribed but is also accurately ligated beginning within the first hour after microinjection (14, 15).

In the study reported here, we utilize the molecular characteristics of the avian retrovirus to study the constraints upon intracellular ligation of microinjected compared with transfected DNA. Upon infection, single-stranded virion RNA is reverse transcribed into linear and then circular double-stranded viral DNA which integrates into the host chromosome (for review, see reference 33). The integrated provirus contains long terminal repeats (LTR) (12, 25) at each terminus which function both as transcription promoters and terminators (Fig. 1) (2, 7, 35). Between LTRs the viral *gag* (group-specific antigen) and *pol* (polymerase) genes precede the *env* (envelope glycoprotein) and *src* genes (31). *env* mRNA is formed by removal of the *gag* (except the extreme 5′ terminus) and *pol* genes from a full-length transcript by nuclear RNA processing (Fig. 1) (10). For this reason, it is not surprising that deletion or insertion of various amounts of DNA within the *pol* region of a cloned viral genome inactivates the *pol* gene in the resulting transcript but does not block splicing to form active *env* mRNA (4, 15, 22).

Our approach was, therefore, to construct a plasmid with the viral sarcoma genome positioned between LTR sequences as in the integrated provirus. Viral RNA would be transcribed from the plasmid DNA after its introduction into cultured avian cells unless the viral genomic sequences had been interrupted by restriction endonuclease cleavage. Ligation would reactivate plasmid DNA cleaved within the *pol* gene region unless a mutation accompanied the ligation. Even mutation, however, would not be expected to block *env* mRNA production from the resulting transcript (15). With this approach, a fundamental molecular difference was discovered in the way transfected and microinjected DNAs are ligated within the avian cell. Intracellular ligation is efficient with both techniques, but only microinjected DNA appears to escape mutation during the process of ligation.

MATERIALS AND METHODS

Cells and culture methods. QT35 cells were obtained (20) from a chemically induced tumor in quail cells. Throughout the course of these studies Rous sarcoma virus (RSV)

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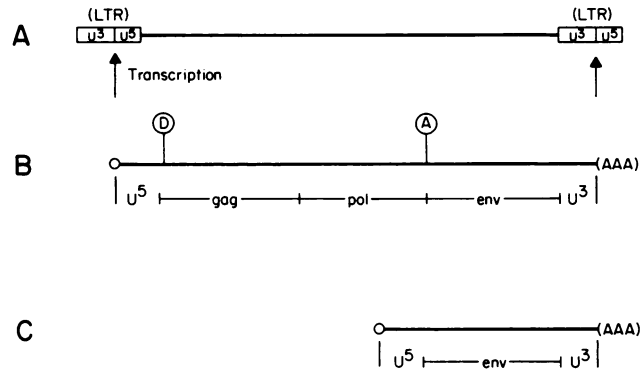

FIG. 1. Retroviral polynucleotides. Linear retroviral DNA (A) contains the viral structural genes located between viral LTR sequences. Transcription initiates and terminates within the LTR (indicated by arrows) to yield full-sized viral RNA (B) containing a 5′ capped nucleotide and polyadenylic acid at the 3′ terminus. The 3′-terminal sequence (U^3) and 5′-terminal sequence (U^5) of the viral RNA molecules make up the DNA LTR sequence. *env* mRNA (C) is formed by nuclear splicing to remove all but the N-terminal 15 bp of the *gag* and *pol* genes from full-length viral RNA. Splice acceptor (Ⓐ) and donor (Ⓓ) sites are indicated by circled letters. Because the 15 bp at the N terminus of the *env* structural gene is joined to the rest of *env* by splicing (10), proper splicing may be required for *env* activity.

helper assays (see below) were used to ensure that these cells were not virus infected before use. Qcl3 cells are quail cells transformed with Bryan RSV by R. Friis and were obtained from M. Linial. These were periodically checked in the focus assay to ensure that they released no transforming virus particles. Focus assays were performed by infecting chicken embryo fibroblasts (CEFs) with one-half of the culture fluid collected for each analysis. Consequently, numbers used throughout this work consistently represent one half of the total released. Conditions for focus assay and CEF culture conditions have been described previously (14). For helper-virus assays, RSV^- cells were infected with the sample to be tested. After 4 days, focus assays were performed to test the presence of transforming virus released by infected RSV^- cells; these would indicate virus in the original sample. Chicken embryos (gs^-, Chf^-) were obtained from SPAFAS (Norwich, Conn.). The infectious center analysis was performed by infecting 4.5×10^5 CEFs on a 35-mm plate with culture fluids in the presence of 8 μg of DEAE-dextran. After 18 h, cells received 10,000 R of X-irradiation, were incubated for 1 h, and were subcultured along with 10^6 untreated CEFs on a 60-mm dish. After 4 h, the cells were overlaid with agar-containing medium (0.9%) and incubated for 7 days. This infectious center assay was slightly modified for analysis of cytoplasmic injections. For this study, pL397 DNA was microinjected into the cytoplasm of 200 CEF or chick RSV^- cells. After 1 h, injected cells were X-irradiated and subcultured with normal CEFs and overlaid as above. Chick RSV^- cells were obtained by Sendai virus-mediated fusion of RSV^- virions and CEFs as previously described (14). Quail cells were cultured in medium 199 (GIBCO Laboratories, Grand Island, N.Y.) with 1% dimethyl sulfoxide, 5% fetal calf serum, 1.25% chick serum, and 10% tryptose phosphate broth. Chick cells were cultured in Scherer medium with 2% calf serum and 10% tryptose phosphate broth.

Microinjection and transfection. Plasmid DNA was microinjected into the nuclei of 150 cells or the cytoplasm of 200 cells. Immediately after injection, the cover slip on which microinjected cells were attached was placed in fresh culture medium. The entire culture fluid (2.0 ml) was collected and frozen at the times indicated. Microinjection techniques have been described previously (28). Glass capillary tubes with outside diameters of near 1.0 μm were mechanically introduced into the cell at the desired location, and the sample was forced into the cell under pressure. Visual observations were used to regulate the amount of sample injected; the amount averaged perhaps 1 to 2% of the cell volume for nuclear injections and 5% for cytoplasmic injections. The micropipette was removed from the cell immediately after injection. DNAs were ethanol precipitated before injection. DNA was dissolved in 0.14 M KCl–10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA at a concentration of 0.1 mg/ml. Approximately 200 molecules were injected into each nucleus at this concentration.

For transfection, 1.5×10^5 cells were plated onto a 35-mm dish. After 20 h, the culture medium was removed and the cells were washed with incomplete medium (quail culture medium lacking dimethyl sulfoxide, tryptose phosphate broth, and sera [27]). DNA was then added to 0.7 ml of incomplete medium along with 200 μg of DEAE-dextran per ml and placed in contact with cells for 4 h. Finally, complete medium was added. Either 232 or 580 ng of DNA was added to each plate. During cotransfection experiments, 232 ng of each DNA type was administered simultaneously.

Restriction enzymes and DNA purification. Restriction enzymes were obtained from New England Biolabs (Beverly, Mass.). Cleavage conditions were those recommended by the supplier. To ensure complete cleavage, restricted DNA preparations were analyzed by agarose gel electrophoresis on a miniaturized gel system as previously described (13). Purification of the DNA fragment was performed by restriction endonuclease cleavage by endonuclease *Kpn*I followed by *Hin*dIII. The DNA was then electrophoresed on a 20-cm, 1% low-melting-point agarose gel (Sea Plaque; FMC Corp., Marine Colloids, Rockland, Maine), and the high-molecular-weight fragment was excised from the gel and purified by two phenol extractions and ethanol precipitation.

Construction of pL397. Parts of two viral plasmids were combined in the formation of pL397 (Fig. 2). Both were originally obtained by cleaving circular viral DNAs from infected cells with restriction nuclease *Sal*I and cloning into pBR322. *Sal*I cleaves the viral DNA within the *env* gene so that the virus-specific regions of the resulting clones are a linear permutation of the viral genome with portions of *env* at each terminus (4). Plasmid pL39td2.4 was obtained from a transformation-defective Schmidt-Ruppin B RSV (SR-B), whereas pLD6 is a nondefective, transforming clone of SR-B (Stacey, manuscript in preparation). pLD6 is similar to pL39td2.4 except for the presence of the *src* gene. The 5.0 kilobase pairs of DNA between *Bam*HI sites in the *gag* and *env* genes of pLD6 was removed by *Bam*HI restriction endonuclease cleavage followed by ligation at high dilution to form plasmid pL6Bh7 (4). The pBR322 sequences between the *Bam*HI and *Hin*dIII sites had previously been eliminated from pLD6. DNAs of plasmids pL39td2.4 and pL6Bh7 were mixed in equimolar amounts and cleaved with endonuclease *Sal*I. The DNA was ethanol precipitated and then ligated at 50 mg/ml. Colonies resulting from transformation of *Escherichia coli* RRI cells with the ligated DNA were separately analyzed by hybridization with nick-translated probes obtained from sequences located only in the *src* gene and from sequences between *Bgl*II restriction sites located in *gag* and *pol*. Clones positive in both screenings were ana-

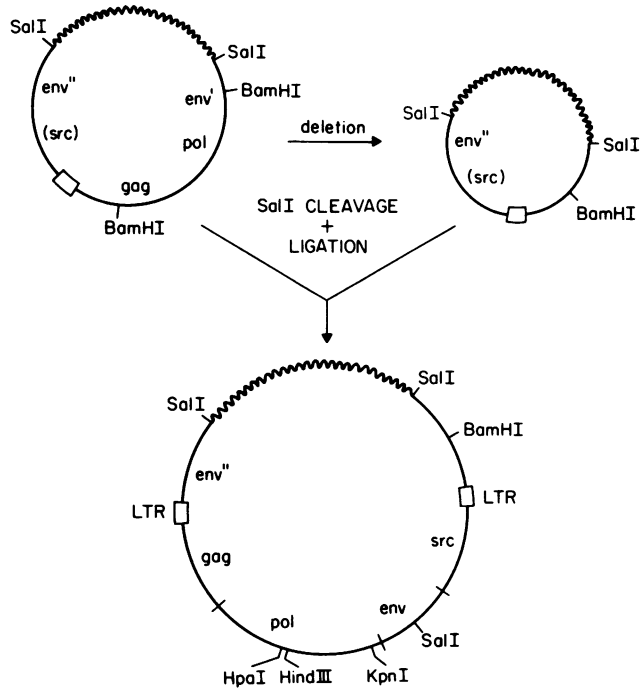

FIG. 2. Construction of plasmid pL397. For the generation of pL397, two viral clones were used. Both contained permuted viral genomes with parts of *env* at each terminus of the virus-specific sequence. pL39td2.4 (upper left) contained the genes *gag*, *pol*, and *env*, whereas pL6Bh7 (upper right) contained parts of *env* and the entire *src* gene. Cleavage of the two plasmids followed by religation at high concentration yielded plasmid pL397 with all four viral genes arranged between viral LTR sequences. Four unique restriction sites are indicated: *Hin*dIII, *Hpa*I, and *Kpn*I in the viral *pol* gene and *Pvu*I (not illustrated) in the pBR322 sequence. (The *Hin*dIII site in pBR322 had previously been eliminated in pL6Bh7.)

lyzed by mini lysate to determine the orientation of the two viral genomes to obtain pL397.

Ligation. In some cotransfection experiments, circular, double-stranded, virus-specific DNA was required. This was formed by cleavage of plasmid pL39td2.4 with *Sal*I followed by ethanol precipitation. The cleaved DNA was then ligated for 18 h at 4°C in the presence of 1 mM ATP and polynucleotide kinase (New England Biolabs) at a DNA concentration of 1 μg/ml. The resulting DNA was analyzed by agarose gel electrophoresis in the presence of ethidium bromide. Between 30 and 50% of all viral DNA appeared to be in the form of a relaxed double-stranded circle by virtue of its increased mobility (which was similar to circular plasmid DNA of the corresponding size). No purification was performed upon this ligated material before cotransfection.

RESULTS

Experimental design. For the study reported here, it was necessary to determine the proportion of molecules ligated and the frequency of mutation at the site of ligation. A plasmid was, therefore, constructed to contain a complete viral genome located between two LTR sequences so that transcription of the intact plasmid would yield full-sized viral RNA. Unique restriction sites located within pBR322 sequences and within the viral polymerase (*pol*) gene were identified. Finally, a biological system was used which could distinguish between correct ligation and ligation accompanied by alteration of the molecular structure. This analysis involved the use of two quail cell lines. QT35 cells were obtained from a chemically induced tumor and express no viral gene products. Qcl3 cells were transformed by Bryan RSV and contain all viral genes except *env*. A viral DNA containing a mutation in *pol*, therefore, would not be expected to yield infectious virus in QT35 cells since the input DNA must provide all viral gene functions. However, within RSV$^-$ cells (Qcl3), this DNA could provide functional *env* mRNA which would complement the deficiency of Bryan RSV leading to the production of infectious, transforming virus by recipient cells. Even in RSV$^-$ cells, viral DNA cleaved within the *pol* gene would have to be rejoined by ligation to restore *env* gene activity, since the cleavage would otherwise physically separate the viral promoter along with the amino terminus of envelope glycoprotein from the remainder of the *env* gene (10).

The plasmid constructed for this study, pL397 (Fig. 2), contains unique *Hin*dIII and *Hpa*I sites located within *pol* ca. 2,000 base pairs (bp) from *env*, whereas a unique *Kpn*I site is located in *pol* ca. 75 bp from the splice acceptor site for *env* (24). Of these three enzymes, only *Hpa*I yields blunt ends. A unique *Pvu*I site is located within pBR322 DNA removed from the viral genomic region by over 2,000 bp and would, therefore, not be expected to affect viral expression. Finally, pL397 encodes a subgroup B envelope glycoprotein. Since quail cells used throughout these studies (unless otherwise stated) are not susceptible to infection by this viral subgroup, little or no virus spread would occur within transfected or microinjected cultures. Virus observed must, therefore, have been produced by initial DNA recipient cells and not those subsequently infected within the treated culture.

Microinjection. Uncut plasmid pL397 DNA or DNA cleaved at one of the four restriction sites listed above was individually microinjected into QT35 and RSV$^-$ cells. Culture fluids were collected from injected cultures and analyzed for the titer of focus-forming units (FFU). Although the results of individual injection experiments varied somewhat due to physical variations in the injections received, no significant differences were observed in the number of FFU released from QT35 cells after injection of any of the DNA preparations, except for the appearance of a slight reduction in virus titer with *Hpa*I-cleaved DNA. This reduction might indicate that blunt-end ligation is inefficient in microinjected cells. Similar results were obtained after microinjection into RSV$^-$ cells (Table 1).

These results indicate that in both cell types transcription from linearized or closed circular DNA was roughly equivalent. Furthermore, DNAs cleaved internally were as efficient as DNA linearized outside the viral sequences when tested in QT35 cells. Thus, we concluded that ligation within the microinjected cell was highly efficient and that little or no alteration of the viral sequences occurred during this ligation.

Transfection. Circular pL397 DNA or DNA cleaved with each of the four restriction enzymes listed was next administered to QT35 or RSV$^-$ cells in the presence of DEAE-dextran. Supernatant fluids were collected for 5 days and analyzed for FFU as described above. In both cell types, intact plasmid DNA or DNA cleaved outside the viral sequences with *Pvu*I yielded similar results, indicating that the linear and circular molecules were taken up by the cells, transported to the nucleus, and subsequently transcribed with nearly equal efficiency. With the RSV$^-$ cells, plasmid DNA cleaved internally with *Hin*dIII, *Kpn*I, or blunt-end-

TABLE 1. Microinjection of pL397 plasmid DNA into QT35 and RSV⁻ cells, intact, or after cleavage with single-cut restriction endonucleases

DNA injected	FFU at the following time after injection:			
	QT35			RSV⁻
	16 h	40 h	64 h	16 h
Cut with restriction enzyme:[a]				
None	65	80	40	
*Pvu*I	52	70	36	82
*Hin*dIII	60	71	100	78
*Kpn*I	44	42	28	
*Hpa*I	11	21	14	
*Hin*dIII-*Kpn*I fragment[b]	0	0	0	0

[a] DNA of plasmid pL397 was cleaved with the indicated enzyme and microinjected into the nuclei of QT35 cells or Qcl3 (RSV⁻) cells. Culture fluids were collected at various times after injection.

[b] pL397 DNA was cleaved with *Hin*dIII and *Kpn*I, and the 14-kilobase-pair fragment was purified by agarose gel electrophoresis and was microinjected (in an experiment described separately in the text).

producing *Hpa*I yielded nearly as many FFU as circular DNA (Table 2). As with microinjection, this result indicated that the transfected DNA was quantitatively ligated. A slight reduction in FFU from RSV⁻ cells transfected by internally cleaved DNA might indicate that ligations were at times multimolecular events able to produce some improperly oriented molecules. Since within RSV⁻ cells the input DNA need produce only *env* mRNA to lead to FFU production, alteration in the DNA at the site of the ligation would not be apparent. In the case of *Kpn*I-cleaved DNA, however, a deletion of over 75 bp at the cleavage site would be likely to involve the *env* splice site or structural gene. The high level of activity with the *Kpn*I-cleaved DNA indicates that any deletions regularly occurring within the input DNA must have been less than 75 bp in length.

Results with QT35 cells contrasted sharply with those described above. There was approximately a 50-fold reduction in the number of FFU released from QT35 cells transfected with DNA cleaved at the *Hin*dIII, *Hpa*I, or *Kpn*I sites (Table 2). Since virus released from QT35 cells requires the expression of all viral genes from input DNA, this result indicates either that a mutation had been introduced into the viral genome or that ligation did not occur efficiently.

To distinguish between these two possibilities, DNA able to provide viral *gag* and *pol* activities was cotransfected into QT35 cells together with restriction endonuclease-cleaved pL397 DNA. If ligation of the cleaved pL397 DNA occurred, transcription within the cell would yield a viral genomic RNA containing *src*. The postulated mutation in the *pol* gene of ligated pL397 DNA should be complemented by the cotransfected DNA to allow at least partial restoration of FFU production from the QT35 cells. Since cotransfected DNA lacked *src*, all FFU must be derived from pL397 DNA which had undergone ligation within the cell. Two forms of complementing DNAs were used in these cotransfections. The first was the lymphoid leukosis viral clone pL39td2.4, which is a permutation of the linear viral DNA with parts of *env* of each terminus of the virus-specific region (Fig. 3 and reference 4). Transcription initiating at the viral LTR would proceed through the *gag* and *pol* genes before termination and has been shown to provide *gag* and *pol* activities after microinjection (unpublished data). The second was this same plasmid DNA, cleaved with *Sal*I and ligated at high dilution (to yield a high proportion of circular viral molecules) before transfection (Fig. 3). Cotransfection of QT35 cells with either of these DNAs resulted in 10 to 25% restoration of the FFU from *Hin*dIII- or *Kpn*I-cleaved pL397 when compared with values obtained with circular or *Pvu*I-cleaved pL397 DNA (Table 3). This result suggested that the pL397 DNA had been ligated. Thus, the low level of FFU observed after transfection with linear pL397 alone probably reflects the presence of a mutation in the *pol* gene.

To further establish that ligation after transfection produces mutations, an infectious center analysis was performed on virus released from transfected cells. To yield an infectious center, an individual virus must express *gag*, *pol*, *env*, and *src*. Mutations at the site of ligations should reduce the number of infectious center-producing viruses for internal viral DNA cleavages but not for circular or *Pvu*I-cleaved pL397. As expected, the number of infectious center-producing viruses produced after transfection into either RSV⁻ or QT35 cells was reduced severalfold when results with intact and virus-cleaved plasmid DNA were compared (data not shown). The fact that in RSV⁻ cells the titer of FFU was

TABLE 2. Transfection of QT35 and RSV⁻ cells with pL397 plasmid DNA, intact, or after cleavage with single-site restriction endonucleases

DNA used	FFU[a]											
	QT35 cells						RSV⁻ cells					
	232 ng of DNA			580 ng of DNA			232 ng of DNA			580 ng of DNA		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Cut with restriction enzyme[b]												
None	3	28	60	6	66	74	7	52	96	29	86	144
*Pvu*I	2	36	50	3	76	88	14	64	131	19	137	152
*Hin*dIII	0	1	0	0	2	7	5	30	96	15	52	134
*Kpn*I	0	0	0	0	3	3	7	59	52	17	115	212
*Hpa*I[c]	0	0	0	0	1	1	1	14	9	2	53	48
*Hin*dIII-*Kpn*I fragment[d]	0	0	0				0	11	45			

[a] Number of days shows the time after transfection when FFU were determined.

[b] DNA of plasmid pL397 was cleaved with the restriction endonucleases indicated and transfected into QT35 or Qcl3 (RSV⁻) cells. Culture fluids were collected and assayed for virus titers thereafter.

[c] Data obtained in a separate experiment.

[d] See Table 1, footnote *b*.

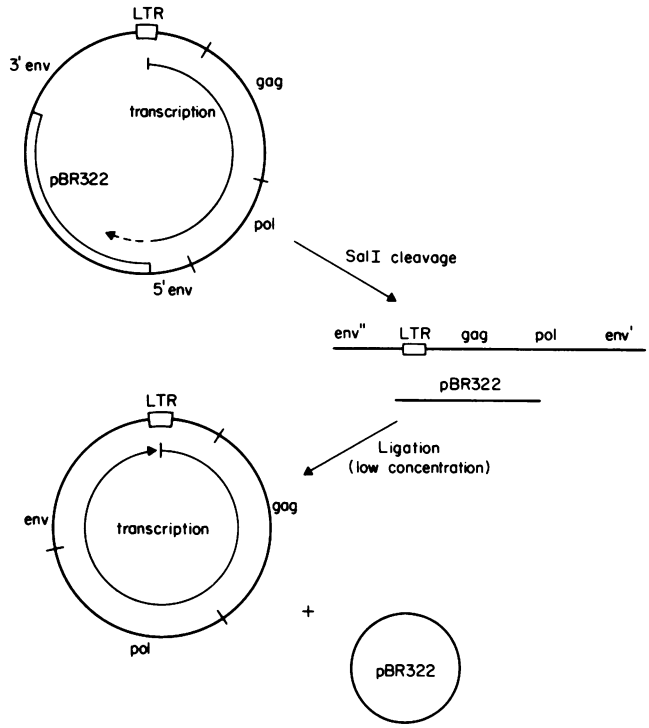

FIG. 3. Lymphoid leukosis molecular clone used in cotransfection. Circular viral DNA cloned into pBR322 at the unique *Sal*I site yields a plasmid containing a permuted *env* gene (upper left). Transcription from this plasmid within a cultured cell would initiate at the LTR and proceed through *gag* and *pol* before termination (as shown by unpublished complementation experiments). When this plasmid is cleaved by *Sal*I and ligated at high dilution, 30 to 50% of the viral DNA forms double-stranded circular viral DNA molecules (lower left), as shown by microinjection (manuscript in preparation), to be transcribed to yield all viral gene products required for virus production.

similar with both types of DNA whereas the titer of infectious center-producing viruses declined with internally cleaved plasmids confirms that ligations do occur with high efficiency but that alterations are normally introduced near the site of ligation.

Ligation of noncomplementary termini. To demonstrate directly the difference in the fate of molecules after microinjection and transfection, we analyzed viral DNA with noncomplementary termini in *pol*. pL397 was cleaved with *Kpn*I and *Hin*dIII and purified by agarose gel electrophoresis. (Independent studies confirmed that if the *Hin*dIII and *Kpn*I termini were joined together, the resulting molecule would be active in *env* mRNA production [4].) As expected, the purified, double-cut molecule was unable to promote FFU production from transfected QT35 cells or from microinjected RSV$^-$ cells. It was, however, active after transfection of RSV$^-$ cells (Tables 1 and 2, *Hin*dIII-*Kpn*I fragment). Apparently, alterations (such as exonuclease cleavage) at the termini of transfected DNA must have rendered them susceptible to ligation, whereas the absence of alteration within microinjected cells left the noncomplementary termini unable to undergo ligation.

Cytoplasmic injections. A final question concerns the time at which mutations are introduced into transfected molecules. Since DNA introduced directly into the nucleus by microinjection does not undergo detectable mutation, it is likely that the alterations occur during transfection either at the time of passage through the cytoplasm or upon entry into the cell. To test this notion, DNA was injected into the cytoplasm. It has been found that cytoplasmic injections are much less efficient than nuclear injections when only a few molecules are injected (14; unpublished data). To compensate for this lower efficiency, a range of DNA concentrations was utilized. Ten-fold dilutions of *Hin*dIII-cleaved pL397 DNA were injected into the nuclei or cytoplasm of RSV$^-$ chicken cells or CEFs. After 1 h, the cells were lethally X-irradiated and subcultured with normal CEFs as in the infectious center assay. Only injected cells able to release infectious transforming virus would induce the formation of a focus. Within RSV$^-$ cells this would simply require *env* gene activity of the injected DNA and should occur regardless of mutation provided that the *Hin*dIII-cleaved DNA was ligated. For an injected CEF to induce a focus, however, ligation must occur without mutation. It was found that CEF and RSV$^-$ cells were equally efficient in yielding infectious centers (Table 4). Therefore, although DNA introduced into the cytoplasm is less likely to be active than DNA introduced directly into the nucleus, those molecules which ultimately were transcribed were not likely to have been altered during ligation. It therefore appears that alterations leading to mutations occur before entry into the cytoplasm (or nucleus) and may involve nuclease action within lysosomes involved in DNA uptake (17).

TABLE 3. Cotransfection of QT35 cells with pL397 DNA along with closed circular lymphoid leukosis viral DNA[a]

Cotransfection	FFU			
	*Hin*dIII	*Kpn*I	*Pvu*I	None
−	0	1	145	88
+	27	17	128	76

[a] pL397 DNA was digested with the enzymes listed and administered to QT35 cells with or without an equal amount (232 ng) of lymphoid leukosis viral DNA in the form of an in vitro-ligated closed circle (Fig. 3). Supernatants were collected at 48 h for analysis. Similar results were obtained when uncut plasmid DNA was used in place of the closed circular lymphoid leukosis viral DNA.

DISCUSSION

In these studies, the molecular fates of microinjected and transfected DNAs have been compared with the aid of retroviral genetics. Both the efficiency and fidelity of ligation

TABLE 4. Cytoplasmic injection of *Hin*dIII-digested pL397[a]

Cell type	No. of foci			
	Nuclear (10)[b]	Cytoplasmic		
		10[b]	100[b]	1,000[b]
CEF	38	0	1	7
RSV$^-$	60	0	1	9

[a] 200 injections of *Hin*dIII-digested pL397 DNA were performed for each experiment with CEF or chick RSV$^-$ cells. The numbers observed with nuclear injections reveal that CEFs survive this procedure less efficiently than RSV$^-$ cells. Although few foci resulted from cytoplasmic injections, results similar to these were consistently observed in a number of experiments. In separate experiments with circular pL397 DNA or linear DNA obtained from infected cells, similar results were obtained (Stacey, manuscript in preparation), indicating that inefficient nuclear accumulation is the property of most DNAs.

[b] Number of molecules of DNA injected per cell.

were determined. A DNA clone was constructed to contain an uninterrupted, nondefective avian sarcoma viral genome arranged between LTR sequences (as in the integrated provirus; Fig. 2). Transcription to yield full-sized viral RNA could occur either from the circular plasmid molecule or from cloned DNA linearized by cleavage within the pBR322 sequences. Plasmid molecules cleaved at one of three unique restriction endonuclease sites within the viral *pol* gene, however, could yield active viral RNA only after ligation to reconstitute the viral genome. Furthermore, for the *pol* gene itself to be functional after such a ligation it is expected that accurate rejoining would be required. Independent studies confirm that even small deletions within viral structural genes inactivate that gene function after introduction into a cultured cell (22). In the case of the *pol* gene, however, even extensive deletions or insertions of DNA do not normally block production of active *env* mRNA (4), since the *pol* gene region is removed as an intervening sequence during splicing to form *env* mRNA.

DNAs of the three types listed, circular plasmid molecules or those linearized outside viral sequences or by cleavage within the viral *pol* gene, were introduced into quail cells. The efficiency of DNA utilization was indicated by the titer of virus released by treated cells. With both methods of DNA introduction, circular molecules or those linearized by cleavage within vector sequences yielded similar virus titers. This indicates that linear and supercoiled molecules are taken up and transcribed with equal efficiency.

Plasmid molecules linearized within *pol* were also highly active after microinjection, whereas this DNA was reduced over 95% in activity after transfection into uninfected quail cells. To demonstrate that this reduction resulted from limited mutations and not from a reduction in the efficiency of ligation, DNA was transfected into RSV^- cells which express all viral genes except *env*. In these cells, input DNA need direct synthesis only of *env* mRNA to lead to virus production. The production of *env* mRNA would require ligation but need not be affected by a mutation in the *pol* gene. Transfected, *pol*-cleaved DNA was fully active in RSV^- cells compared with other forms of DNA. To further establish this point, transforming virus titers were significantly increased when circular nontransforming viral DNAs, able to provide viral structural gene functions, were cotransfected along with *pol*-cleaved plasmid DNAs. In this case, ligation of the *pol*-cleaved plasmid would be required to account for the production of transforming viral genomic RNA, since transforming genomic molecules would not be transcribed from a bisected viral genome.

As a further demonstration of the distinction between ligation after transfection and after microinjection, a deletion was introduced into the *pol* gene of the plasmid described above by cleavage with two unique restriction endonucleases followed by purification of the resulting deleted genome. Restriction enzymes were chosen to generate noncomplementary, protruding, single-stranded termini. Separate molecular constructs verify that if the two termini were joined the resulting deletion in *pol* would not affect *env* mRNA production and therefore virus production from recipient RSV^- cells (unpublished data). After transfection, the deleted genome yielded near-normal virus titers from RSV^- cells, whereas no virus production was observed after microinjection. Cellular enzymes had apparently generated termini able to be ligated only in transfected cells. Even polymerase activity to fill in single-stranded regions would have generated blunt termini known to be ligated. (*Hpa*I-cleaved DNA, with blunt termini, was efficiently ligated in this work.) The absence of an enzymatic system which readily alters termini of DNA molecules within the cellular nucleus may be of importance in DNA repair or recombination.

The *Kpn*I restriction site is located within the *pol* gene, 75 bp from the *env* splice acceptor site. Deletions extending more than 75 bp would have inactivated *env* and eliminated activity of *Kpn*I-cleaved DNA even after transfection into RSV^- cells, which was not observed to occur. Apparently, deletions must generally be limited in length. It is conceivable that exonuclease action on a dextran-treated DNA conglomerate might encounter steric limitations to the degradation of large DNA segments. Although endonuclease activity is also likely to be a factor in transfection, it would not be apparent in these comparative studies since circular or linear molecules would probably be inactivated to similar extents.

Differences in the fate of DNA introduced by microinjection and transfection must relate to the molecular mechanism by which transfected DNA enters the cells. It appears that in transfection DNA first enters lysosomes and escapes into the cytoplasm before entering the nucleus (17). Since DNA is active essentially immediately after microinjection into the nucleus or cytoplasm (14), the delay in transcription of nearly 24 h after transfection provides an estimate of the length of time required for transit into the cell. The mutations introduced during transfection do not appear to occur during transit through the cytoplasm, however, since linear DNA injected directly into the cytoplasm is equivalent in activity to circular DNA. This analysis neglects the fact that most DNA injected into the cytoplasm apparently never enters the nucleus for reasons which are not presently understood. The important fact is that those few DNA molecules which did enter were efficiently ligated without detectable mutation.

Folger et al. (6) have analyzed DNA microinjected into mammalian cells after that DNA had become integrated into the host genome. Consistent with this work, over 90% of the ligations they observed occurred without mutation. In a separate experiment, however, they conclude that soon after injection DNA molecules were joined regardless of their molecular termini. Although this result involves different cell types and a different biological assay, it contrasts with results described here, in which microinjected DNAs were ligated only at complementary termini and without mutation. In addition, we have conducted other studies to determine the efficiency of joining of multiple purified retroviral DNA fragments injected into avian cells. The results clearly establish that noncomplementary termini are rarely if ever joined (15).

These results also reveal a significant difference between DNA metabolism in cultured cells and in amphibian oocytes. In these studies, cultured cells transcribed with equal efficiency DNA introduced as circular or linear plasmid DNA. This was the case with either microinjection or transfection. In contrast, recent studies with amphibian oocytes reveal that DNA injected as a linear molecule is much less efficiently transcribed than circular DNA (11).

Finally, Miller and Temin (19) recently analyzed ligation of separate fragments of retroviral DNA transfected into avian cells. Consistent with the work reported here, the separate fragments were only 1% as active as full-sized viral DNA or fragments ligated in vitro before transfection. The assay used, however, determined the limiting amounts of transfected DNA needed to generate a single virus-producing cell within the culture. The authors concluded that under

these circumstances virus production was determined by the probability that an individual cell would take up two biologically active fragments. In the work reported here, the amounts of viral DNA transfected were constant and the termini to be ligated were located on the same molecule so that probability considerations involved in transfection were not a factor in our results.

The observation that transfected DNA molecules are often altered (16, 26, 34) as well as efficiently joined together (21, 23) is well established. The significance of the work described here is the demonstration that ligation of microinjected DNA occurs rapidly and efficiently and does not involve mutation. In addition, DNA introduced as a linear molecule is transcribed as efficiently as a circular molecule. In addition to this work, other studies indicate that only complementary single-stranded DNA termini (or blunt termini) are ligated after microinjection (15). These facts demonstrate a fundamental distinction between the molecular structures obtained after transfection or microinjection of DNA. This observation is likely to be of importance in the design of experiments aimed at assessing the biological properties of many cloned DNAs. These studies also provide insight into the metabolism of free DNA molecules within a living cell.

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