essentially unchanged. Similarly, B1 and F2 turn by 87°, while B2 and F1 remain unchanged. The rest of the molecules, including the pairs C, D, G, and H, are essentially unaffected, none exhibiting rotation of the steroid nucleus by more than 4°. There is little change in any of the side chains, except those of C2 and G1, whose conformation changes from *gauche-trans* to the energetically more stable (*16*) *trans-trans* and whose terminal isopropyl groups become disordered.

The mechanism of the transition can be understood by considering intermolecular packing contacts made by the rotated and unrotated molecules and by the side chains of molecules C and G. Molecule C in the room-temperature and C1 in the body-temperature form have the energetically unfavorable *gauche-trans* side chain conformation. This is a consequence of crowding by their neighbor molecules B, notably by the methyl group (C21) at the base of the side chain (Fig. 1). Rotation of B1 allows the side chain of C2 to extend to *trans-trans*. However, the rotated B1 interferes with molecule A in its room-temperature orientation; this interference is, in turn, relieved by the rotation of A1. Of the eight possible combinations for the three molecules:

C: *gauche-trans* (C1) or *trans-trans* (C2)

B: rotated (B1) or unrotated (B2)

A: rotated (A1) or unrotated (A2)

only the two actually present, namely C1, B2, A2 and C2, B1, A1, give acceptable intermolecular packing. It is noteworthy that neither combination interferes with molecule D, which explains why this molecule remains unaffected. By the pseudosymmetry present in the structure (see below), the same mechanism applies to molecules G, F, and E. The ability of molecules to turn about their long axes without disrupting the overall crystalline packing and the multitude of different lateral packing contacts exhibited by the steroid nuclei (Fig. 1, end views) support a biomembrane model involving random, relatively nonspecific lateral interactions (*9*).

Closely obeyed rotational or translational pseudosymmetry exists in the crystal structure of the anhydrous room-temperature form (*14*), as well as in the monohydrate (*17*) and the hemiethanolate (*18*) of cholesterol. The molecular reorientations and the onset of side chain disorder in the phase transition are all related by one set of twofold pseudoscrew axes present in the anhydrous room-temperature form (Fig. 1). The result is that every other axis of pseudosymmetry is preserved in the transition, while every other one is destroyed. The preserved axes are very closely obeyed. A rotation of 180.3° about an axis at the center of the diagram (Fig. 1, top), almost parallel to **c**, accompanied by a translation of approximately **c**/4 superposes the 224 atoms of the left half of the cell (molecules B1, A1, . . . , D2, C2) onto the right half (molecules F2, E2, . . . , H1, G1) with a mean atom-atom misfit of only 0.3 Å, side chains included.

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19. Symbols: **a**, **b**, and **c** are the unit cell axes; α, β, and γ are the angles between the unit cell edges; Z is the number of molecules per unit cell; and ρ is the density.
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High-Efficiency Ligation and Recombination of DNA Fragments by Vertebrate Cells

Abstract. *DNA-mediated gene transfer (transfection) is used to introduce specific genes into vertebrate cells. Events soon after transfection were quantitatively analyzed by determining the infectivity of the DNA from an avian retrovirus and of mixtures of subgenomic fragments of this DNA. The limiting step of transfection with two DNA molecules is the uptake by a single cell of both DNA's in a biologically active state. Transfected cells mediate ligation and recombination of physically unlinked DNA's at nearly 100 percent efficiency.*

Studies of stable transformants after specific genes have been introduced into cells have shown that physically unlinked DNA's can be transferred into the same cell and become physically linked to one another (*1*). Studies of DNA soon after transformation show that intramolecular blunt-end ligation is as efficient as intramolecular homologous cohesive-end ligation (*2*) and that the efficiency of intramolecular cohesive-end ligation is approximately 10 percent (*3*).

When two DNA molecules are used in DNA-mediated gene transfer (transfection) experiments, intermolecular ligation and recombination can occur. In studies of transfection with two overlapping SV40 DNA fragments, the infectivity of the two fragments was 0.1 percent (*4*), or 1 percent (*5*) of the infectivity of circular SV40 DNA. After transfection of cells with both SV40 DNA and an unrelated, physically unlinked DNA, recombination occurred in 1 percent of the cells that replicated SV40 DNA (less than 1 percent of all cells) (*6*). The number of cells receiving both DNA's, and thus the efficiency of cell-mediated joining of input DNA's, was not determined.

We have carried out experiments designed to determine the efficiency of cell-mediated joining of DNA's and the limiting step of cotransfection experiments. Our results show that cells physically join unlinked DNA's at close to 100 percent efficiency and that the limiting step of cotransfection is the ability of a single cell to take up both DNA's in a biologically active state.

For cotransfection experiments, we used mixtures of subgenomic DNA clones from spleen necrosis virus (SNV) and looked for production of infectious virus. Spleen necrosis virus is an avian retrovirus that is highly pathogenic in vivo (*7*). In cell culture SNV causes cell death during acute infection (*8*), although some cells survive and multiply while continuing to produce virus (*9*). The introduction of SNV DNA into susceptible

cells by transfection leads to production of infectious virus (*10*). The infectivity of the cloned DNA was assayed by the appearance of cytopathic effects and of DNA polymerase activity and was quantified by end-point dilution. For the end-point dilution assay, 1 μg of DNA was serially diluted and added to four dishes of chicken embryo fibroblasts for each dilution. Each plate was scored for the presence or absence of virus production. The amount of DNA added to each plate at the dilution producing virus in 50 percent of the plates is equivalent to one infectious unit.

Cell-mediated ligation was studied by cotransfection of cells with subgenomic clones of SNV DNA that form a complete SNV DNA molecule upon ligation (Fig. 1). Transfection by a single subgenomic clone led to no detectable infectious virus production (data not shown) (Table 1). Furthermore, none of the mixtures of subgenomic clones (which do not contain homologous regions) was infectious when circular molecules were used for cotransfection, indicating that in these cases recombination did not generate full-length SNV DNA (data not shown) (Table 1).

In the study of ligation in a noncoding region, subgenomic clones of SNV that were cloned at the Sal I site (p14-44 5′ RI-Sal I and p14-44 3′ Sal-RI, p60B Sal) were digested with Sal I and introduced into chicken embryo fibroblasts. In the study of ligation in coding region, subgenomic clones that were cloned at the Hind III site (p14-44 5′ RI-Hind III and p14-44 3′ Hind III-RI) were digested with Hind III and introduced into chicken embryo fibroblasts. The infectivity of these mixtures of molecules with cohesive ends was approximately 1 percent of the infectivity of full-length SNV DNA (p14-44) (Table 1). Ligation was observed in both noncoding and coding regions at approximately the same efficiency. When mixed Sal I subgenomic clones were digested with Sal I and transferred into canine thymus cells (Cf2Th) the infectivity was also 1 percent of the infectivity of full-length SNV DNA (data not shown), showing that this ligation phenomenon is not specific to chicken cells. The infectivity of subgenomic clones that have been digested as described above and then ligated in vitro before transfection was as high as the infectivity of full-length SNV DNA (data not shown). This result shows that the difference in infectivity between the subgenomic clones and full-length SNV occurs because only some subclone molecules are ligated in vivo, and not because ligated molecules are less infectious than complete molecules.

Blunt-end ligation was studied by digesting the Sal I subclone molecules with Sal I and then with S1 nuclease to hydrolyze single-stranded regions. The infectivity of these molecules with blunt ends was approximately 0.3 percent of the infectivity of full-length SNV DNA (Table 1). However, during the preparation, more than 50 percent of the DNA was lost (data not shown). Therefore, the infectivity of the molecules with blunt ends was probably similar to that of molecules with cohesive ends. Thus cells can ligate molecules with blunt ends as efficiently as those with cohesive ends. The infectivity of subgenomic clones digested with Sal I, treated with S1 nuclease, and ligated in vitro was as high as that of full-length SNV DNA (data not shown).

Physical evidence of ligation of the input DNA was obtained by analysis (*11*) of unintegrated linear DNA of virus produced by the transfected cells (*12*). The DNA of virus produced after transfection of cells with Sal I subgenomic clones that were digested with Sal I and S1 nuclease had lost the Sal I restriction

Table 1. Infectivity of mixtures of DNA subclones of spleen necrosis virus (SNV). Relative infectivity is the ratio of the infectivity of the tested DNA to the infectivity of p14-44, multiplied by 100. The infectivity of one infectious unit of p14-44 is 6.7×10^{-6} pmole. At least three experiments were performed to determine the infectivity of each tested DNA unless otherwise indicated. Infectivity between experiments varied on the average by a factor of 4.

DNA	Treatment	Relative infectivity
p14-44	None	100
p14-44 5′ RI-Hind III	None	< 0.01
p14-44 3′ Hind III-RI	None	< 0.01
p60B Sal	Sal I	0.6
p14-44 5′ RI-Hind III + p14-44 3′ Hind III-RI	None	< 0.01
p14-44 5′ RI-Hind III + p14-44 3′ Hind III-RI	Hind III	4
p14-44 5′ RI-Sal I + p14-44 3′ Sal-RI	Sal I	1
p14-44 5′ RI-Sal I + p14-44 3′ Sal-RI	Sal I + S1	0.3
p14-44 5′ RI-Sal I + p14-44 3′ Sal-RI	Sal I + separate precipitation	0.2*
p14-44 5′ RI-Sal I + pREV-A Sal-Hind III + p14-44 3′ Hind III-RI	Sal I + Hind III	0.1*
p14-44 5′ RI-Hind III + p14-44 3′ Sal-RI	None	1
p14-44 5′ RI-Sal I (not diluted) + p14-44 3′ Sal-RI	Sal I	100

*Single experiment.

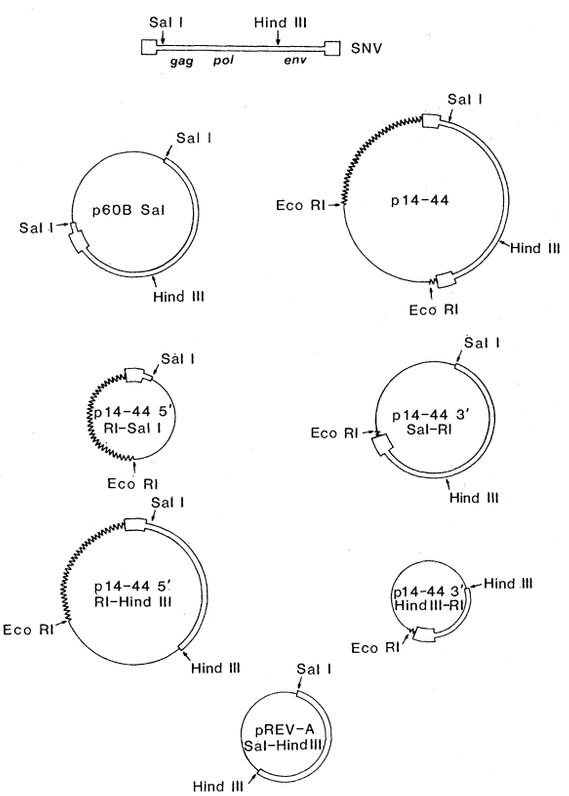

Fig. 1. Map and subclones of the DNA of spleen necrosis virus (*SNV*). The map shows the three genes and relevant restriction endonuclease cleavage sites of SNV. The Sal I site is in a noncoding region; the Hind III site is in a coding region. The DNA subclones have been described (*17*). Two subclones (see text for specific pairs) were mixed in equal amounts, and the mixture was digested with enzymes as described in the text, serially diluted in Hepes buffer containing sheared salmon sperm DNA (10 μg/ml, except where indicated in the text), and precipitated with calcium phosphate (except where indicated in the text). Each dilution (0.5 ml) was added to each of four 60-mm dishes of chicken embryo fibroblasts (*18*). Double lines represent SNV sequences; open boxes, long terminal repeats; jagged lines, cell sequences; and single lines, plasmid pBR322 sequences.

Table 2. Infectivity of TK-SNV and helper virus (REV-A) DNA's. Infectivity is measured both as picomoles of DNA required for virus production in 50 percent of the plates (one infectious unit) [pmole (IU)] and as picomoles of DNA required for production of thymidine kinase colonies in 50 percent of the plates (one thymidine kinase activity transforming unit) [pmole (TKTU)]. The ratio of the TKTU value to the IU value is given. The infectivities are averages from three separate experiments. Infectivity between experiments varied by a factor of 4, but relative infectivity between experiments varied only by a factor of 2.

Treatment	Infectivity		Ratio
	pmole (IU)	pmole (TKTU)	
Precipitated together	6.7×10^{-6}	7.2×10^{-4}	100
Precipitated separately	6.7×10^{-6}	7.2×10^{-3}	1000

endonuclease cleavage site. The DNA of virus produced after transfection of cells with Sal I subgenomic clones digested with Sal I alone was a mixture in which most of the population (at least 85 percent) retained the Sal I cleavage site (data not shown). This result indicates that cell-mediated ligation is often perfect (resulting in no loss of bases), but sometimes includes limited degradation and thus loss of an enzyme cleavage site. In extending these findings, Sal I subgenomic clones from molecular clones of SNV that differ in several restriction endonuclease cleavage sites were digested with Sal I and used to cotransfect cells. The DNA of the resultant virus contained the expected mixture of parental restriction endonuclease sites (data not shown), thus proving that two separate molecules were ligated together.

The efficiency of multiple ligations were studied by cotransfecting cells with three subgenomic clones (p14-44 5′ RI-Sal I, pREV-A Sal-Hind III, and p14-44 3′ Hind III-RI) that were digested with Hind III and Sal I [the helper virus REV-A has 98 percent nucleotide sequence homology with SNV (*13*)]. Two ligation events are needed to produce a full-length SNV molecule. The infectivity of this mixture of three subgenomic clones was 0.1 percent of the infectivity of full-length SNV DNA (Table 1). Although the efficiency of one ligation event is apparently 1 percent, the efficiency of two ligations is ten times that expected for individual ligation events occurring at random.

To determine whether ligation may be enhanced by the coprecipitation of separate DNA fragments in calcium phosphate, we either mixed the Sal I subgenomic clones (digested with Sal I) before the dilutions and precipitations (the usual procedure) or separately diluted and precipitated the subgenomic clones and then mixed them when they were added to cells. The infectivity of subgenomic clones precipitated together was 1 percent of the infectivity of full-length SNV DNA, and the infectivity of subgenomic clones precipitated separately was 0.2 percent of the infectivity of full-length SNV DNA (Table 1). Thus, when the DNA's are precipitated separately, the infectivity is approximately one-fifth that of DNA's precipitated together. Although the DNA's may be brought into proximity by the coprecipitation step, coprecipitation of separate DNA's does not appear to be a major limiting step of cotransfection.

The efficiency of homologous recombination was studied by cotransfection with subgenomic clones containing overlapping sequences. The infectivity of subgenomic clones containing 5 kilobase pairs of overlapping sequences (p14-44 5′ RI-Hind III and p14-44 3′ Sal I-RI) was approximately 1 percent of the infectivity of full-length SNV DNA (Table 1). The form of these molecules did not affect the infectivity: circular, linear, and concatenated molecules were equally infectious (*14*). When clones containing identical long terminal repeats and an additional 40 base pairs (bp) of overlapping sequences further downstream were used for cotransfection, recombination occurred within the 40-bp region (not quantified, data not shown). When cells were cotransfected with clones containing only 6 bp of overlapping sequences and no long terminal repeats in common (14-44 Sal I or Hind III 5′ and 3′ subgenomic clones), no recombination was seen. Thus, when the length of overlapping sequence is short, the long terminal repeats, because of their 600-bp homology, may have a role in aligning the molecules so that recombination can occur.

These results confirm that the infectivity of virus fragments is 1 percent of the infectivity of complete virus DNA in tranfection experiments, as determined for SV40 (*5*). To extend these findings, we determined the limiting step of cotransfection. Because all of these assays were done by diluting both DNA's, the results could reflect the ability of a cell to take up both types of DNA in a biologically active state rather than the ability of a cell to ligate two DNA molecules. To test this possibility, we used two separate DNA clones to cotransfect chicken embryo fibroblasts, a clone of a replication-defective retrovirus containing the thymidine kinase gene of herpes simplex virus (TK-SNV) and a clone of replication-competent helper virus REV-A (*15*). The DNA's were either mixed before dilution and precipitation or diluted and precipitated separately and then mixed. Replication-competent virus is produced by cells that take up and express the helper virus DNA, whereas TK-SNV is produced only by cells that take up and express both DNA's. This assay, thus, distinguishes between cells that take up and express only one DNA and those that take up and express both DNA's. The virus produced 5 days after transfection was passaged to fresh chicken embryo fibroblasts to assay for helper virus (*8*) and to buffalo rat liver cells deficient in thymidine kinase to assay for thymidine kinase–transforming units. The infectivity of TK-SNV DNA was 1 percent of the infectivity of helper virus DNA after coprecipitation and 0.1 percent after separate dilutions and precipitations (Table 2). Thus, only a small percentage of the cells that take up and express REV-A virus DNA also take up and express TK-SNV DNA, even after coprecipitation. This 100-fold difference is the same as the difference between infectivity of mixtures of subgenomic clone molecules and of full-length SNV seen above. The tenfold difference between separate precipitations and coprecipitation in this experiment is similar to the difference seen with subgenomic clone molecules. These results indicate that at limiting DNA concentrations the limiting step of cotransfection is the uptake of two DNA's in a biologically active state by a single cell.

A further test of the ability of cells to ligate input DNA is to dilute serially one subclone and use the other subclone in excess amounts (10 μg/ml) instead of sheared salmon sperm DNA as carrier. The infectivity of this mixture was approximately 100 percent of the infectivity of full-length SNV DNA (Table 1). This result indicates that the efficiency of intermolecular ligation is approximately 100 percent.

Our results indicate that the limiting step of these cotransfections is the ability of cells to take up two physically unlinked DNA's in a biologically active state. When a cell takes up two DNA's the efficiency of joining (both ligation and recombination) is very high. It is not yet known whether the cellular enzymes

involved in this activity are induced by introduction of DNA into the cell. However, studies of microinjection of DNA fragments into cells have shown that intermolecular recombination and ligation occur at a very high efficiency (*16*), indicating that this phenomenon may not be specific to transfection. One use of this joining activity is the ability to construct recombinant DNA molecules in vivo. If selection exists for a desired recombinant molecule, the molecule can be constructed in vivo by cotransfection of the appropriate DNA fragments. These findings also indicate that caution must be used in designing transfection experiments. Studies of DNA after transfection will be studies of complex molecules, not linear molecules, because of the high efficiency of ligation and recombination.

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Inhibition of Gastric Acid Secretion in the Gastric Brooding Frog, *Rheobatrachus silus*

Abstract. *The female gastric brooding frog* Rheobatrachus silus *broods its young in its stomach. A substance that inhibits gastric acid secretion in a toad stomach preparation in vitro appears to be secreted by the developing young. This substance has been identified as prostaglandin* E_2. Rheobatrachus silus *may thus have developed a mechanism whereby prostaglandin secreted by the larvae inhibits acid secretion in the stomach of the female until the larvae have completed development and emerged as juvenile frogs by way of the female's mouth.*

In southeast Queensland there is a rare aquatic frog, *Rheobatrachus silus*. The female frog swallows fertilized eggs or early-stage larvae and broods the young in her stomach. The young frogs eventually emerge by way of her mouth (*1*, *2*). During the period of gastric brooding, the gastric musculature undergoes modification and the oxyntic cells show ultrastructural features typical of the absence of acid secretion (*3*). Oral birth of the young frogs occurs at least 8 weeks after they are ingested; the gastric ultrastructure returns to normal a few days later. Investigators have speculated that the young release a substance that inhibits gastric acid secretion. Because the species is near to extinction, our data in this and previous reports (*1*–*3*) are based on studies of only five female frogs collected over 8 years. Our studies have been performed on histological specimens or on secretions obtained during the short life-span of these females and their young in captivity. Here we provide evidence that *R. silus* tadpoles secrete a substance that inhibits the secretion of gastric acid by an isolated amphibian gastric mucosal preparation. High concentrations of this inhibitory substance, which was identified as prostaglandin E_2 (PGE_2), were present in aquarium water inhabited by young *R. silus* immediately after their premature birth.

In December 1978 a female *R. silus* expelled 24 early-stage tadpoles while being transferred to an aquarium. Twenty-two of these larvae were transferred to an open plastic container of purified, softened tapwater. The tapwater had been filtered through sand to remove particulate matter, through activated carbon to dechlorinate it, and through a fine micropore filter to exclude large bacteria. The water was aerated gently and maintained at 30°C. Tapwater for the related, control species *Limnodynastes tasmaniensis* was treated in the same way.

Copious, fine cords of mucus were released from the mouths of the tadpoles. Because an inhibitor of gastric acid secretion might be associated with the mucus, the aquarium water, which was replaced daily, was collected and stored at −20°C. Before being assayed, this water was concentrated by freeze-drying; 500 ml of water produced about 0.24 g of solid material that was redissolved in 10 ml of Mackenzie's solution (*4*).

We used the spontaneously secreting gastric mucosa of *Bufo marinus* as the principal bioassay for the potential inhibitor of gastric secretion. The toads were pithed, the stomach was removed, and the gastric mucosa was dissected from the muscle coats. Fundic mucosa was mounted as a diaphragm between two halves of a Lucite chamber to provide exposed mucosal and serosal surfaces measuring 2.0 cm^2. The serosal surface was bathed in 12 ml of Mackenzie's solution, a buffered salt solution for *Bufo marinus* tissues (*4*). The mucosal surface was bathed in 12 ml of isotonic NaCl. Both solutions were aerated and circulated by gas lifts of 95 percent O_2 and 5 percent CO_2. The mucosal solution was held at *p*H 6 by titration with 0.05*M* NaOH in a *p*H stat system (Radiometer Copenhagen). This system provided a measure of the rate of H^+ secretion which was expressed as microequivalents per square centimeter per hour. The test solution was applied to the serosal side of the mucosal preparation.

The isolated mucosa of *Bufo marinus* secreted acid spontaneously at a rate of 1.92 ± 0.69 $\mu Eq/cm^2$-hour (mean ± standard deviation, $N = 49$). The rate was independent of the sex of the toad and remained relatively constant for at least 3 hours. In each of six experiments, the addition of 1.5 ml of the concentrated aquarium water to the gastric mucosal preparation decreased the rate of spontaneous acid secretion. The mean rate (± S.D.) of acid secretion 30 minutes before the addition of the aquarium water was 1.88 ± 0.54 $\mu Eq/cm^2$-hour; during the second 30-minute period of exposure the mean rate was 0.28 ± 0.24 $\mu Eq/cm^2$-hour (Fig. 1). A similar degree of inhibition was effected by $10^{-6}M$ PGE_2, $10^{-6}M$ somatostatin, and $10^{-4}M$ cimetidine. In two experiments, the inhibitory effect of aquarium water was