



## Stereochemical Course of Catalysis by the Tetrahymena Ribozyme

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the kernels were classified into types by a cluster analysis method and use of nearest centroid sorting. To select the best number of groups, the cluster analysis was run four times specifying different numbers of clusters (6, 8, 10, and 12). Ten clusters corresponded most closely to the types perceived subjectively. The attributes of the ten types are given in Table 1. These archeological types should not be understood as representing actual races of maize; they are rather an initial classification for assessing the relative variation through time.

Types C, F, and G are small, mostly beaked kernels that fall in the size range of the modern Andean popcorn races (2, 3). Type I is a short, thick type with a low row number (8-row). Types A, D, and E are larger, long in proportion to their width, with quite high row (12- to 18-row) numbers and many square-capped kernels. Types H and J are large broad kernels, with rounded caps, flat cross sections, and low row (8- and 10-row) numbers.

The relation among the ten types can be seen in a plot of the first and second canonical variables, which together account for 86% of the variability in the assemblage (Fig. 2). The first canonical variable (increasing width, length, and relative flatness) is associated with time, as is shown in the bar graphs below the scatterplot in Fig. 1. The two earliest assemblages are dominated by small type F. The Pancán 2 assemblage appears to be more varied, although the sample size is small. Greater variation and domination by a new type (type C, a high row number beaked type) is evident in level 1 of Pancán. Diversity in types is greatest in Wanka II and Wanka III, each phase with kernels of all ten types, though in different proportions. Two unusually large type H kernels found in Wanka II contexts approach the size and shape of the Cuzco races, suggesting that large-kerneled, low-row number varieties were grown in the Mantaro before Inca times, rather than being spread with Inca hegemony as has been suggested (2).

On the coast of Peru researchers have noted, after a long and rather stable period dominated by popcorns, a sudden change toward new flint and flour maize types from the highlands (2, 3). They postulate that this change is associated with the Wari expansion out of the Ayacucho region (Middle Horizon, about A.D. 550 to 850). The Mantaro Valley data indicate that an influx of new flint and flour types there is not associated with Middle Horizon, but rather with the Late Intermediate (after A.D. 1000). This maize evidence parallels the lack of Middle Horizon Wari architecture and the scarcity of Wari sherds in the area in

indicating that the people of the upper Mantaro received little direct Wari influence.

At the close of the Middle Horizon in the Mantaro area a number of changes in ways of growing, preparing, and eating food are associated with the new use of large-kerneled maize types. Stone hoes become more common (15), as do the actual remains of corn and other crops (15, 21). Large grinding stones and manos become more common in relation to mortars and pestles (15), perhaps reflecting grinding of the new floury maize types. Bowls become larger in Wanka I times, with new vessel types appearing in Wanka II and in Inca times (15). This may reflect new foods suitable to floury maize, such as *chicha* (maize beer), *mote* (hominy), *kancha* (toasted maize), and *sanco* (fine cornmeal cakes). Maize in Inca times, and probably earlier, was a high-prestige and ceremonial food (22).

Comparison of charred maize kernels from six time periods has helped in understanding changes in maize use and associated culture in the Mantaro Valley over a millenium. The data show (i) a continuity in use of small-sized maize into and through the Middle Horizon, indicating a lack of Wari influence there as compared to the coast, and (ii) a florescence of large-kerneled flint and flour types occurring in the Late Intermediate, associated with new cultural dimensions in farming, crops, and eating.

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## Stereochemical Course of Catalysis by the *Tetrahymena* Ribozyme

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**The group I intron from *Tetrahymena* catalyzes phosphodiester transfer reactions on various RNA substrates. A modified RNA substrate with a phosphorothioate group in one stereoisomeric form at the site of reaction was synthesized in order to determine the stereochemical course of an RNA-catalyzed reaction. The reaction product was digested with a stereospecific nuclease to determine the configuration of the product phosphorothioate. The reaction occurs with inversion of configuration at phosphorus, implying an in-line pathway for the reaction.**

**T**HE GROUP I SELF-SPICING RIBOSOMAL RNA intron from *Tetrahymena* catalyzes its excision from the primary RNA transcript and exon ligation to form the mature transcript (1, 2). In vitro, the free intron can function as a site-specific ribonuclease, a terminal transferase, a phosphotransferase, and an acid phosphatase, depending on the RNA substrate provided

and the conditions of the reaction (3–5). This RNA is a true catalyst in that it can mediate multiple turnovers of some reactions (3, 6).

Whether the ribozyme catalyzes transesterification reactions stereospecifically has

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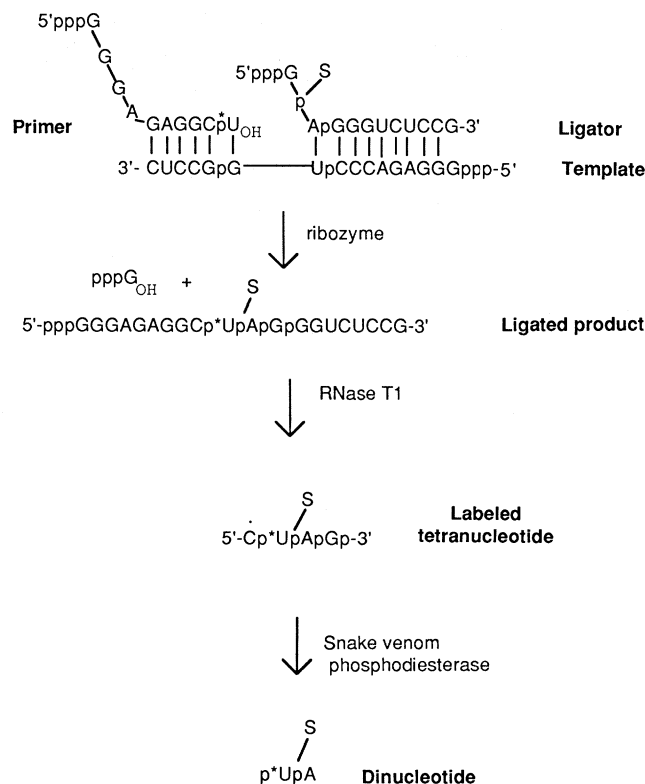
not been determined even though the stereochemical course of such reactions can provide evidence for particular catalytic mechanisms. Many protein enzymes that catalyze similar reactions of nucleic acid substrates have been studied with phosphorothioate analogs (one of the peripheral oxygens of a phosphate ester is replaced by sulfur) that allow the stereochemical course of the reaction to be determined. All protein enzymes that have been investigated stereochemically catalyze nucleophilic displacements at phosphodiester bonds with inversion of the configuration at phosphorus (7, 8).

We initially attempted to determine the stereochemistry of the RNA-catalyzed reaction with our previously described substrate cleavage assay (9, 10). However, the phosphorothioate analog turned out to be an extremely poor substrate for the cleavage reaction, and we were unable to obtain a large enough sample for analysis. We have found that a template-directed oligonucleotide ligation reaction occurs with much greater efficiency than the cleavage reaction, with up to several hundred turnovers per enzyme molecule (11). We therefore decided to use this system to examine reaction stereospecificity.

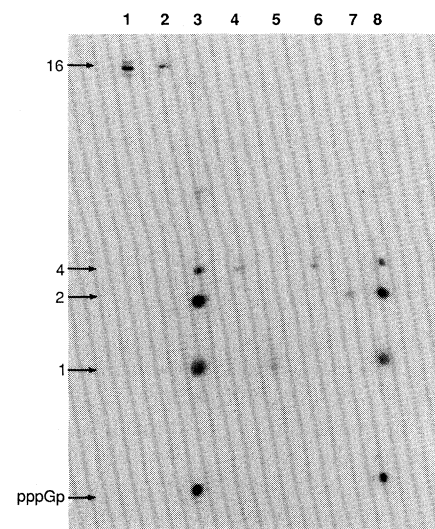
A combination of primer, template, and ligator oligonucleotides was designed such that the reactive phosphate could be specifically substituted with a phosphorothioate. This phosphate is the 5' phosphate of the

single adenosine residue in the ligator oligonucleotide (Fig. 1). The RNA's were synthesized by T7 RNA polymerase transcription of synthetic deoxyoligonucleotides (12). For a control experiment with no phosphorothioate, the primer was labeled with [ $\alpha$ - $^{32}$ P]rGTP (ribosyl guanosine triphosphate), and the purified RNA's were tested for ligation by the intron. After incubation, the primer was almost completely incorporated into a 20-nucleotide product, which comigrated with a synthetic RNA of the same sequence. The identity of the ligated product was confirmed by RNase (ribonuclease) T1 digestion; the digestion pattern was identical to that of the control RNA.

The ligator RNA was then synthesized with rCTP, rGTP, rUTP (ribosyl cytidine, guanosine and uridine triphosphate), and  $\alpha$ S-rATP (adenosine triphosphate) ( $S_p$  isomer). Since T7 RNA polymerase catalyzes polymerization with inversion of configuration at each phosphate, the phosphorothioate group is in the  $R_p$  isomeric form once it is incorporated into the RNA (13). The primer was labeled with [ $\alpha$ - $^{32}$ P]rUTP. After ligation of the phosphorothioate analog to the primer, the phosphorothioate group should be  $S_p$  if the reaction occurs with inversion (for example, by an associative in-line mechanism),  $R_p$  if the reaction proceeds with retention (by an adjacent associative mechanism with pseudorotation), or a mixture of  $R_p$  and  $S_p$  if the reaction occurs by a



**Fig. 1.** Scheme for determining the stereoselectivity of phosphodiester bond transfer. The ribozyme-catalyzed ligation reaction was carried out in 10 mM  $\text{NH}_4\text{Cl}$ , 20 mM  $\text{MgCl}_2$ , 30 mM tris-HCl, pH 7.4, 1 mM aurin tricarboxylic acid, and 10  $\mu\text{M}$  primer, ligator, template, and enzyme RNA's in a 50- $\mu\text{l}$  reaction volume. The reaction was incubated for 3 hours at 58°C, and then stopped by the addition of an equal volume of 90 percent formamide containing 1 mM EDTA. The product was purified and processed as described for Fig. 2.



**Fig. 2.** Autoradiogram of a denaturing 20 percent polyacrylamide gel on which products of the snake venom digest were isolated. Control and ligated product RNA's were purified on denaturing 20 percent polyacrylamide gels, eluted in 0.3M NaCl, extracted with phenol, precipitated, and resuspended in distilled water. Portions of each were digested to completion with RNase T1, resulting in a single  $^{32}\text{P}$ -labeled tetranucleotide with the sequence CUAG (Fig. 1). The tetramers were gel-purified, concentrated on DEAE columns, eluted in TEAB buffer, dried at reduced pressure, and resuspended in distilled water. The tetramers were then digested with snake venom phosphodiesterase, and the products were separated by electrophoresis on a 20 percent denaturing acrylamide gel. (Lane 1) Control RNA, undigested; (lane 2) ligated product RNA, undigested; (lanes 3 and 8) RNase T1-digested control RNA (uniformly [ $\alpha$ - $^{32}$ P]rGTP-labeled) for size markers; (lane 4) tetramer isolated from RNase T1-digested product RNA; (lane 5) snake venom digest of the control tetramer; (lane 6) tetramer isolated from RNase T1-digested product RNA; (lane 7) snake venom digest of the product tetramer.

dissociative mechanism in which the leaving group is first expelled and then followed by nucleophilic attack at either face of the planar metaphosphate intermediate (7, 8). As in the cleavage reaction, the presence of sulfur at the phosphorus undergoing substitution inhibited the reaction. However, when the concentration of RNA enzyme was increased from 0.2  $\mu\text{M}$  to 10  $\mu\text{M}$  and the incubation time was extended from 1 hour to 3 hours, approximately 5 percent of the primer was ligated into the expected phosphorothioate-containing product.

A control RNA with the same sequence as the product RNA was synthesized by T7 RNA polymerase transcription of a synthetic oligonucleotide, with  $\alpha$ S-rATP and [ $\alpha$ - $^{32}$ P]rUTP. In this RNA the phosphorothioate group is in the  $R_p$  configuration. The control RNA and the ligated product RNA were gel-purified and each was digested to completion with ribonuclease T1 to yield CpUpApGp, in which the first phosphate is

labeled with  $^{32}\text{P}$  and the second is a phosphorothioate. The sequence of the tetramer from the control RNA was confirmed by nearest neighbor analysis. Each tetranucleotide was digested with snake venom phosphodiesterase (SVPDE), which has a 1700-fold greater activity on  $R_p$  isomers (8, 13), and the products were separated by gel electrophoresis (Fig. 2). The control RNA ( $R_p$  isomer) was digested to mononucleotides, with less than 1 percent of the  $^{32}\text{P}$  in the dinucleotide region. The ligated product RNA was only digested to the labeled dinucleotide pUpA. Quantitation of the gel on a  $\beta$ -scanner (Betagen) showed that less than 2 percent of the radioactivity was in mononucleotides. The inability of the phosphodiesterase to cleave the dinucleotide to mononucleotides indicates that the phosphorothioate product is  $S_p$ , and therefore that the ribozyme reaction proceeds with inversion of configuration. The experiment was repeated with ( $\alpha$ - $^{35}\text{S}$ )-labeled rATP to label the ligator and control RNA's, and the same result was obtained: the phosphorothioate in the ribozyme-generated RNA was resistant to phosphodiesterase cleavage, while the control RNA was digested to mononucleotides.

Replacing the pro- $R$  oxygen of the reactive phosphate with sulfur decreases the rate of reactions by a factor of about 1000. One possible explanation for this effect is that the larger sulfur interferes with the coordination of a  $\text{Mg}^{2+}$  ion that is normally bound to the two phosphate oxygens, where it functions either in substrate binding, in stabilizing the transition state, or in charge neutralization to facilitate the nucleophilic attack. This rate decrease notwithstanding, it is clear from a number of cases where the stereochemical course of enzymic reactions has been studied both by the use of  $^{16}\text{O}$ ,  $^{17}\text{O}$ , or  $^{18}\text{O}$  phosphodiester groups and by the use of a phosphorothioester, that the elemental substitution does not affect the stereochemical consequence at phosphorus (8, 14).

Any odd number of nucleophilic substitution reactions at phosphorus can result in inversion of configuration of the phosphorothioate (7, 8). In the simplest scheme, a single displacement reaction occurs in which the enzyme catalyzes the nucleophilic attack of the 3' hydroxyl of the primer on the first internal phosphate of the ligator, resulting in product formation. The 5'-guanosine residue of the ligator is released. In the transition state of such a reaction the phosphorus has trigonal bipyramidal geometry, with incoming and leaving groups in the axial positions.

This mechanism implies an in-line orientation of the incoming and leaving hydroxyl groups. It seems likely that this mechanism

will apply to all of the phosphotransfer reactions catalyzed by the ribozyme. If so, the guanosine that attacks the P1 stem in the first step of self-splicing must be oriented by the enzyme so that its O3' is opposite the O3' of the leaving exon. This is an important constraint in attempts to model the active site of the enzyme.

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## Identification of the Fusion Peptide of Primate Immunodeficiency Viruses

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**Membrane fusion induced by the envelope glycoproteins of human and simian immunodeficiency viruses (HIV and SIV<sub>mac</sub>) is a necessary step for the infection of CD4 cells and for the formation of syncytia after infection. Identification of the region in these molecules that mediates the fusion events is important for understanding and possibly interfering with HIV/SIV<sub>mac</sub> infection and pathogenesis. Amino acid substitutions were made in the 15 NH<sub>2</sub>-terminal residues of the SIV<sub>mac</sub> gp32 transmembrane glycoprotein, and the mutants were expressed in recombinant vaccinia viruses, which were then used to infect CD4-expressing T cell lines. Mutations that increased the overall hydrophobicity of the gp32 NH<sub>2</sub>-terminus increased the ability of the viral envelope to induce syncytia formation, whereas introduction of polar or charged amino acids in the same region abolished the fusogenic function of the viral envelope. Hydrophobicity in the NH<sub>2</sub>-terminal region of gp32 may therefore be an important correlate of viral virulence in vivo and could perhaps be exploited to generate a more effective animal model for the study of acquired immunodeficiency syndrome.**

**T**HE ENVELOPE GLYCOPROTEINS OF SIV<sub>mac</sub> (gp120 and gp32) are derived from a gp160 precursor through endoproteolytic cleavage and contain all determinants necessary for host cell infection and syncytium formation (1). The gp32, like the gp41 of HIV-1 (2), is the transmembrane glycoprotein (3) that is expressed on the surface of infected cells and incorporated in the viral membrane; gp32 anchors gp120, which contains the binding site for the CD4 antigen (4, 5). The smaller size of the SIV<sub>mac</sub> transmembrane glycoprotein (gp32 as opposed to HIV-1 gp41) is due to the presence of a premature termination codon in the SIV<sub>mac</sub> *env* gene (6); some data indicate that the region after the termination codon is expressed in vivo in SIV<sub>mac</sub>-infected animals (7).

Several studies indicate that the hydrophobic NH<sub>2</sub>-terminus of the transmembrane glycoprotein is primarily responsible for the membrane fusion events involved

with HIV and SIV<sub>mac</sub> infection and with syncytium formation. This region has amino acid sequence similarity to the fusion peptides of ortho- and paramyxoviruses (8), and it has been shown, as for other enveloped viruses, that the cleavage of the *env* precursor resulting in exposure of the gp41 NH<sub>2</sub>-terminus is a necessary event for HIV infectivity (9). Furthermore, insertion mutagenesis performed on this region of the HIV-1 gp41 (inserting four to six amino acids after position 6 of the gp41) abolishes the capacity of the envelope glycoproteins to induce syncytia in a heterologous expression system (4).

The fusion peptides of ortho- and para-

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