

DNA-Catalyzed Formation of Nucleopeptide Linkages**

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Covalent linkages between nucleic acids and amino acid side chains are important in many biological contexts. For example, phosphodiester bonds between the 5'-end of DNA or RNA and the side chain of tyrosine are formed during topoisomerase activity^[1,2] and in genomes of picornaviruses, such as poliovirus,^[3] and an RNA 5'-terminus is linked to a serine side chain of the tumor-suppressor protein p53.^[4] In these and other cases, the ability to form nucleopeptide linkages will enable biochemical experiments that require conjugation of nucleic acids and proteins. Although chemical methods for nucleopeptide synthesis have been devised, the routes are often lengthy, and synthesis of larger nucleoproteins is quite challenging.^[5] A more biologically inspired approach to formation of nucleopeptide linkages seeks enzymes that can directly and specifically create the intended bonds, without requiring orthogonal protecting groups and multistep fragment-assembly pathways. Towards this goal, we considered whether deoxyribozymes (DNA enzymes)^[6,7] might be applied to form nucleopeptide linkages. We also envisioned that identifying deoxyribozymes which form nucleopeptide linkages would expand the scope of reactions known to be catalyzed by DNA.

The first artificial deoxyribozyme (DNA enzyme) was identified by *in vitro* selection in 1994.^[8] Although many deoxyribozymes have subsequently been discovered, most are restricted to catalyzing reactions of oligonucleotide functional groups. RNA cleavage is the most common DNA-catalyzed activity;^[8,9] our laboratory has focused on RNA ligation.^[7,10] Deoxyribozymes that phosphorylate or cap DNA oligonucleotides using ATP have been identified,^[11] as have DNA enzymes that ligate, cleave, or deglycosylate DNA,^[12] cleave a phosphoramidate bond within DNA,^[13] photochemically cleave a DNA thymine dimer,^[14] or metalate a porphyrin.^[15] A fundamental yet unanswered question is the extent to which DNA can catalyze reactions of functional groups that are not normally part of oligonucleotides. For example, no known deoxyribozyme catalyzes the reaction of an amino acid

side chain in any context. With the long-term objective of deoxyribozyme-catalyzed nucleopeptide synthesis, here we addressed the immediate goal of understanding the ability of DNA to catalyze covalent modification of amino acid side chains. We performed *in vitro* selection experiments that assess DNA-catalyzed formation of nucleopeptide linkages involving the side chains of tyrosine, serine, and lysine (Tyr, Ser, and Lys).

We previously reported 7S11 and related deoxyribozymes that create 2',5'-branched RNA in a three-helix-junction (3HJ) architecture by catalyzing the reaction of a branch-site RNA 2'-OH group with an RNA 5'-triphosphate.^[16–19] Here we sought to reveal the intrinsic catalytic ability of DNA with amino acid side chains while avoiding the need for a discrete peptide binding site, either by design^[20] or by selection. Therefore, we separately placed each potentially reactive amino acid Tyr, Ser, and Lys at the intersection of the 3HJ formed from candidate deoxyribozyme sequences and two nucleic acid strands (Figure 1). One of these strands is a

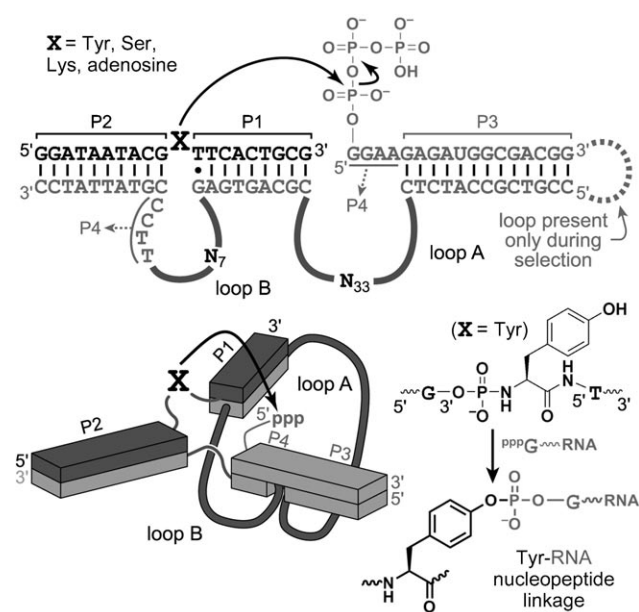


Figure 1. Three-helix-junction (3HJ) architecture derived from the 7S11 deoxyribozyme^[16–19] for presenting amino acid side chains to 5'-triphosphate-RNA for DNA-catalyzed formation of a nucleopeptide linkage. Loops A and B began the selection process as completely randomized (N₃₃ and N₇). Lower right: The key structure of the DNA-X-DNA substrate (X = Tyr), and the Tyr-RNA phosphodiester linkage within the intended ligation product. Note the T-G wobble pair immediately to the 3'-side of X. The DNA substrate nucleotide adjacent to X was T because only the 5'-NH₂-T DNA phosphoramidite used to prepare each DNA-peptide-DNA strand is readily available,^[23] and we used an existing deoxyribozyme pool strand that has G across from this position.

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DNA-peptide-DNA conjugate that presents its single amino acid side chain to the 5'-triphosphate of the second strand, which is RNA.^[21] At the outset of selection, loops A and B were entirely randomized, with a combined total of 40 random nucleotides. As a positive control, in a parallel selection experiment we used a DNA-rA-DNA substrate strand, which presents its riboadenosine (rA) 2'-OH group as a nucleophile to the 5'-triphosphate.^[22] As a negative control we used a DNA-Ala-DNA substrate, in which alanine does not present any nucleophile that could attack the 5'-triphosphate-RNA.

A deoxyribozyme with the intended catalytic activity will attach the DNA-peptide-DNA strand to the 5'-triphosphate-RNA substrate by a phosphodiester (Tyr, Ser) or phosphoramidate (Lys) nucleopeptide linkage. This covalent attachment enables us to use our previously established selection strategy^[10] to identify active DNA sequences.^[23] Briefly, the 5'-triphosphate-RNA substrate was covalently joined at its 3'-end by T4 RNA ligase to the 5'-end of the DNA pool, for which the total number of random nucleotides was 40 (Figure 1). This represented an expansion of loop A from N₁₅ to N₃₃ relative to our recent selections that used the 7S11 architecture;^[18,24] we reasoned that the more challenging activity of amino acid side chain activation might require the larger random region. During the key selection step, incubation with the DNA-X-DNA strand (50 mM CHES, pH 9.0, 150 mM NaCl, 2 mM KCl, 40 mM MgCl₂, 37°C, 2 h) should lead to a polyacrylamide gel electrophoresis (PAGE) shift only for those candidate deoxyribozyme sequences that successfully joined the amino acid or adenosine moiety to the 5'-triphosphate-RNA. The gel-shifted material was isolated and amplified by PCR, and a new selection round was initiated by attachment of the 5'-triphosphate-RNA using T4 RNA ligase.

For the DNA-rA-DNA positive control selection, a band corresponding to ligation of the adenosine 2'-OH group to the 5'-triphosphate was first discernable beginning at round 6, and robust ligation activity was achieved by round 9 (23%; Figure 2). The deoxyribozymes from this particular selection experiment form the expected 2',5'-branched linkage as assessed by partial alkaline hydrolysis; the most active clone forms the branched DNA-RNA product in 61% yield at 2 h with $k_{\text{obs}} = 0.0094 \text{ min}^{-1}$ at 40 mM Mg²⁺ and pH 9.0.^[23] The success with this positive control experiment validated that our selection approach is capable of providing a high level of ligation activity in the 3HJ architecture when the nucleophilic moiety is embedded within the DNA-X-DNA context.

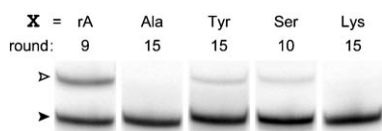


Figure 2. Key rounds for the individual selection experiments with DNA-X-DNA substrate. The images are from 8% PAGE of the indicated round for each selection. The filled arrowhead marks the DNA-X-DNA substrate, and the open arrowhead marks the ligation product. See Figure 1 for the structural context of X, and see the Supporting Information for details of the selection procedure.

Therefore, the results with the DNA-peptide-DNA substrates were anticipated to provide meaningful information on the DNA-catalyzed reactivity of amino acid side chains.

In the DNA-Tyr-DNA selection, ligation activity was first detected at round 12 and leveled off at about 6% by round 15. For the DNA-Ser-DNA selection, the approximately 2% activity achieved by round 10 did not increase upon performing additional rounds. For both the DNA-Lys-DNA and DNA-Ala-DNA efforts, no activity was detected by round 15. We cloned and characterized deoxyribozymes from the Tyr and Ser selections.

From round 15 of the Tyr selection, a survey of the clones revealed two distinct types of catalytic activity. The first type of activity was found for 15 out of 17 clones that share a common sequence named Tyr1; this activity was assigned to formation of the intended Tyr-RNA nucleopeptide linkage. The second type of activity was found for only 2 out of 17 clones that both have the sequence named Tyr13; this activity was assigned to formation of linear DNA-Tyr-DNA-RNA by unanticipated reaction of the DNA-X-DNA 3'-OH group with the 5'-triphosphate-RNA. The activities of both Tyr1 and Tyr13, which have no recognizable sequence homology,^[23] were examined in more detail.

The Tyr1 deoxyribozyme was active with the DNA-Tyr-DNA substrate but none of DNA-X-DNA for which X = Phe, Ala, Ser, Lys, or rA (Figure 3a), consistent with formation of a Tyr-RNA phosphodiester bond. With 40 mM Mg²⁺ at pH 9.0 and 37°C, the Tyr1 yield was as high as 69% at 20 h with $k_{\text{obs}} = 0.0017 \text{ min}^{-1}$, and $K_{\text{d,app}}$ for Mg²⁺ was $(62 \pm 14) \text{ mM}$.^[23] Although not included during the selection process, Mn²⁺ in place of Mg²⁺ was particularly effective as a Tyr1 cofactor, with 72% yield at 2 h and $k_{\text{obs}} = 0.06 \text{ min}^{-1}$ at 20 mM Mn²⁺, pH 7.5, and 37°C ($K_{\text{d,app}}$ for Mn²⁺ of $(12 \pm 3) \text{ mM}$).^[23] Of the other divalent metal ions tested (all at pH 7.5, 37°C), 1 mM Zn²⁺ and 10 mM Ni²⁺ or Cd²⁺ did not support activity, whereas 10 mM Co²⁺ led to a trace amount of ligation product (ca. 0.7% at 20 h; $k_{\text{obs}} = 6 \times 10^{-6} \text{ min}^{-1}$). The uncatalyzed (background) rate constant k_{uncat} was estimated as $< 4.2 \times 10^{-8} \text{ min}^{-1}$ with 40 mM Mg²⁺ or $< 1.3 \times 10^{-7} \text{ min}^{-1}$ with 20 mM Mn²⁺. In both cases, the value of k_{uncat} is an upper limit that is based on failing to detect any product with the DNA-Tyr-DNA substrate and the random DNA pool under the selection conditions ($< 0.01\%$ in 40 h with Mg²⁺ or $< 0.03\%$ in 40 h with Mn²⁺). Therefore, the Tyr1 deoxyribozyme has a rate enhancement conservatively estimated as $> 4 \times 10^4$ (Mg²⁺) or $> 5 \times 10^5$ (Mn²⁺).^[25] The Tyr1 ligation product was isolated by PAGE and analyzed by MALDI-TOF mass spectrometry; the mass was as expected for the assigned Tyr-RNA nucleopeptide linkage (m/z calcd 11660; found 11659 ± 12).^[23] However, mass data cannot distinguish various possible constitutional isomers. Treatment of the Tyr1 product with snake-venom phosphodiesterase 1 (SVPD), which is well known to cleave Tyr-RNA phosphodiester linkages,^[1,3,26] gave degradation products that are consistent with (but do not prove) the assigned structure (Figure 4a).

In contrast to the tyrosine requirement of Tyr1, the Tyr13 deoxyribozyme was highly active with each of DNA-X-DNA where X = Tyr, Ala, Ser, rA, and dA (Figure 3b), indicating that X does not provide the reactive nucleophile for Tyr13. A

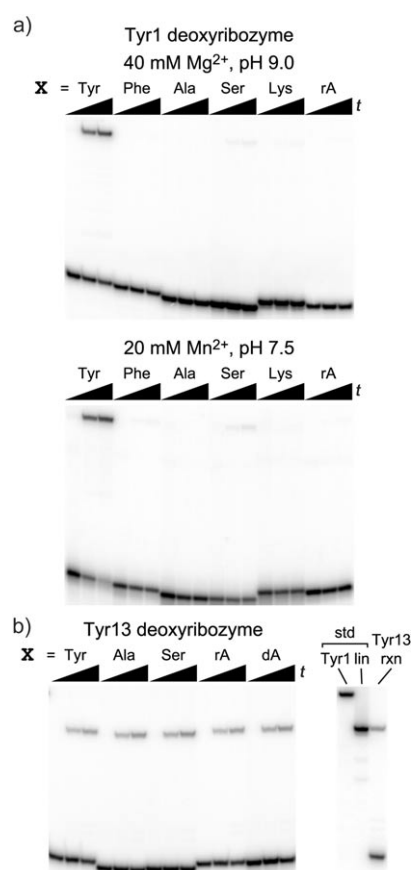


Figure 3. Activities of the Tyr1 and Tyr13 deoxyribozymes. The identity of X (see also Figure 1) is shown above each set of three time points t . a) Tyr1 deoxyribozyme, which forms the intended Tyr-RNA nucleopeptide linkage. Upper image: $t = 0, 5, 10$ h (50 mM CHES, pH 9.0, 150 mM NaCl, 2 mM KCl, 40 mM $MgCl_2$, 37°C). Lower image: $t = 0.5, 20, 60$ min (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, 20 mM $MnCl_2$, 37°C). Only X = Tyr leads to efficient ligation activity. A trace amount of activity is observed with X = Ser, but with k_{obs} lower than for X = Tyr by a factor of 350 (Mg^{2+}) or 200 (Mn^{2+}); the yields were 0.14% in 5 h (Mg^{2+}) and 4.0% in 2 h (Mn^{2+}). b) Tyr13 deoxyribozyme, which forms the unanticipated linear DNA-RNA linkage, contrary to the 3HJ design. The left image shows that X can be any tested amino acid or nucleotide, as expected for reaction of the 3'-OH group that is remote to X ($t = 0, 5, 10$ h; 40 mM $MgCl_2$ as above). The right image shows that the linear product (lin) migrates faster than the topologically branched Tyr1 product, which was purified from the Mg^{2+} reaction of panel (a), but co-migrates with the linear standard prepared by joining DNA-Tyr-DNA with 5'-phosphate-RNA using a DNA splint and T4 DNA ligase. CHES = 2-(cyclohexylamino)ethanesulfonic acid, HEPES = 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid.

3'-phosphate at the terminus of the DNA-dA-DNA substrate completely abolished product formation,^[23] supporting the assigned linear DNA-X-DNA-RNA connectivity. Also consistent with its linear nature, the Tyr13 product migrated substantially faster on PAGE relative to the topologically branched Tyr1 product. The reaction of the 3'-OH group of the DNA-X-DNA substrate as catalyzed by Tyr13 suggests that the overall 3HJ architecture is not maintained. We propose that for Tyr13 complexed with its substrates, the short P4 region is not formed and loop B does not participate in

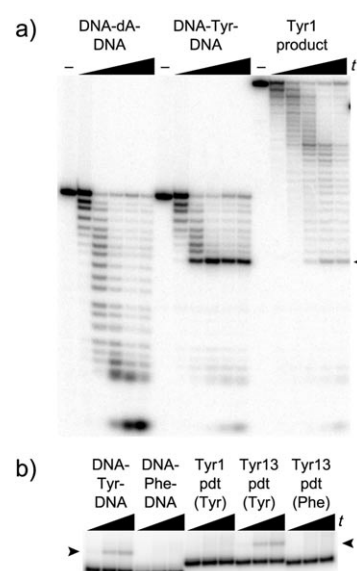


Figure 4. Characterization of the Tyr1 deoxyribozyme product to demonstrate its Tyr-RNA nucleopeptide linkage. a) Treatment with snake-venom phosphodiesterase 1 (SVPD), which cleaves DNA, RNA, and Tyr-RNA linkages (pH 8.0, 4°C, $t = 0.5, 2, 10, 30, 120$ min; –, no SVPD added). The DNA-Tyr-DNA standard and the Tyr1 product (made using DNA-Tyr-DNA as substrate) are predicted to form the same DNA-Tyr-dT product upon SVPD treatment, as observed (arrowhead; SVPD cannot cleave the Tyr-dT amide linkage). In contrast, the DNA-dA-DNA standard is completely degraded, as expected. b) Reaction with TAMRA-NHS after RNase T1 pretreatment. The DNA-X-DNA (X = Tyr or Phe) substrates were used as positive and negative controls for reaction with TAMRA-NHS. As additional positive and negative controls, the Tyr13 linear products (pdt) made using the same two DNA-X-DNA substrates were incubated with TAMRA-NHS. All products were pretreated with RNase T1 to remove the RNA portion before incubation with 2.5 mM TAMRA-NHS (pH 7.0, room temperature, $t = 0.5, 30, 60$ min). Note that reaction was observed only when the Tyr OH group was free (arrowheads); as expected, reaction was not observed for the Tyr1 product.

catalysis (see Figure 1). Consistent with this, mutation of either the P4 portion or the loop B portion of Tyr13 (or deletion of both DNA portions) had little effect on activity.^[23] Both the branched and linear products (of Tyr1 and Tyr13 respectively) survived the selection procedure because the 8% PAGE that was used to separate the active DNA sequences cannot resolve these isomeric products when they are covalently attached to the deoxyribozyme strand; such attachment is required by the selection strategy (see the covalent loop at the upper right of Figure 1). The DNA-rA-DNA selection did not lead to any deoxyribozymes that form a linear product, indicating that formation of such a product is not an inherent outcome of the selection process.

To provide additional evidence regarding the linkages in the Tyr1 and Tyr13 products, we used selective chemical reaction with the tyrosine OH group to assess the absence or presence of the phenol functional group. This hydroxy group is absent in the Tyr-RNA product from Tyr1 because the tyrosine side chain is part of a Tyr-RNA phosphodiester linkage, but the OH group is present in the linear Tyr13 product. By reaction of the Tyr1 and Tyr13 products with the

N-hydroxysuccinimide ester of tetramethylrhodamine (TAMRA-NHS), which has sufficient mass to induce an observable gel shift, we established that the Tyr1 product lacks the phenolic OH group that was present in the DNA-Tyr-DNA starting material, whereas the Tyr13 product retains this functional group (Figure 4b). These data corroborate our key conclusion that the Tyr1 deoxyribozyme forms a Tyr-RNA nucleopeptide linkage.

When complexed with the DNA-Tyr-DNA substrate, the Tyr1 deoxyribozyme has a T-C mismatch adjacent to the Tyr, rather than the T-G wobble pair present at the outset of selection (Figure 1; presumably the G in the deoxyribozyme strand of Tyr1 became mutated during an unidentified PCR round by Taq polymerase). We investigated the role in Tyr1 activity of this mismatch. Restoration of the T-C mismatch to a T-G wobble pair reduced k_{obs} by only threefold with Mg^{2+} and 1.5-fold with Mn^{2+} .^[23] Switching to a T-T mismatch did not affect the ligation activity, whereas introducing a T-A base pair affected activity approximately the same as for T-G. These data suggest that the details of DNA-DNA structural interactions close to the reactive Tyr moiety play only a modest role in successful nucleopeptide linkage formation.

We also examined deoxyribozymes from the Ser selection. The best clone, Ser7, provided merely ca. 0.2% ligation in 5 h at 40 mM Mg^{2+} and pH 9.0.^[23] The activity did not improve at higher Mg^{2+} , and no activity was detected with Mn^{2+} (we previously used Mg^{2+} to select for RNA ligase deoxyribozyme that either do^[17,27] or do not^[28] show increased activity with Mn^{2+}). The Ser7 ligation product migrates on PAGE at approximately the same position as the DNA-Tyr-DNA product for Tyr1,^[23] suggesting that Ser7 creates the analogous Ser-RNA nucleopeptide linkage and not a linear DNA-Ser-DNA-RNA product. Attempts to optimize Ser7 activity by partial randomization and reselection did not lead to any improved deoxyribozymes (data not shown).

From comparison of the robust nucleopeptide linkage formation by the Tyr1 deoxyribozyme, the extremely modest catalysis by Ser7, and the undetectable reactivity during selection of the lysine side chain, we conclude that the phenolic OH group of Tyr facilitates its DNA-catalyzed nucleophilic reactivity. Very few nucleic acid enzymes have been reported to catalyze reactions of amino acid residues (including either the N- or C-terminus), and none have described the reaction of an amino acid side chain.^[29,30] In one case, Baskerville and Bartel identified a Mg^{2+} -dependent ribozyme that joins the free N-terminus of a polypeptide to the ribozyme's own 5'-terminal triphosphate.^[30] Consistent with our findings, their study suggested that the side chains of Ser and Lys are relatively unreactive with nucleic acid catalysts; however, they did not test Tyr.

In summary, we have shown that DNA has the ability to catalyze efficient formation of a Tyr-RNA nucleopeptide linkage between the side chain of tyrosine and a 5'-triphosphate-RNA. The reaction occurs when these functional groups are placed in an appropriate structural context, such as the three-helix-junction architecture previously established for the 7S11 deoxyribozyme. However, the analogous reactions of serine and lysine side chains are very inefficient and undetected, respectively. Based on these findings, our ongoing

efforts towards nucleopeptide linkages are proceeding in several directions. First, we are seeking to understand the conditions under which improved DNA-catalyzed reactivity of Ser and possibly Lys side chains can be obtained. Second, we are exploring in more detail the permissible structural contexts (for example, structural interactions near the key functional groups) for DNA-catalyzed reaction of amino acid side chains. Third, we are aiming to expand the applicability of DNA catalysis to larger polypeptide and protein substrates.

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- [21] Preparation of the DNA-peptide-DNA conjugates by solid-phase synthesis is described in the Supporting Information.
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