Oligonucleotide-directed peptide synthesis in a ribosome- and ribozyme-free system

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Peptide bond formation by the ribosome requires 23S rRNA and its interaction with the 3′-CCA end of tRNA. To investigate the possible evolutionary development of the peptidyl transfer reaction, we tried to obtain peptide bond formation without the ribosome or rRNA simply by using a piece of tRNA—an aminoacyl-minihelix—mixed with sequence-specific oligonucleotides that contained puromycin. Peptide bond formation was detected by gel electrophoresis, TLC analysis, and mass spectrometry. Peptide synthesis depended on sequence complementarity between the 3′-CCA sequence of the minihelix and the puromycin-bearing oligonucleotide. However, proximity of the reacting species was not by itself sufficient for peptide bond formation. In addition, imidazole as a catalyst was required. Its role may be similar to the recently proposed mechanism, wherein A2451 of 23S rRNA works as a general base. Thus, peptide bond formation can be achieved with a simple, minimized system that captures the essence of an interaction seen in the ribosome.

The two domains of tRNA interact with distinct rRNAs in the full ribosome (1). The anticodon-containing domain of tRNA interacts with 16S rRNA in the small ribosomal subunit, whereas the minihelix domain interacts with 23S rRNA in the large subunit. The minihelix is thought to be the more ancient, historical domain of tRNA, and 23S rRNA may also be the more ancient part of rRNA, perhaps coevolving with the minihelix domain. Thus, modern peptide bond formation may have derived from an interaction between an aminoacyl-minihelix and the primitive ribosome. Indeed, we showed that an aminoacyl-minihelix was an efficient substrate in the peptidyl transfer reaction with the 23S rRNA-containing 50S ribosome (2).

Noller *et al.* (3) established that protein-depleted 23S rRNA had peptidyl transferase activity. The structures of the large ribosomal subunit from *Haloarcula marismortui* (4) and its complexes with substrate analogs (5) showed no protein side-chain atoms closer than about 18 Å to the peptide bond being synthesized. These results suggested that 23S rRNA itself has transferase activity. The structure of the *H. marismortui* ribosome with the trinucleotide puromycin (Pm) construct—CCdA-p-Pm—was consistent with the possibility that N3 of A2486 (A2451 in the *Escherichia coli* 23S rRNA) functions as a general base. This suggestion arises because there are no other functional groups closer than 5 Å to the nascent peptide bond (5). Also, dimethyl sulfate (DMS) modification studies indicated that this adenine has a near-neutral pKa (6). However, despite attempts by several laboratories, peptidyl transferase activity has never been detected with protein-free 23S rRNA (7). In addition, no pH-dependent DMS reactivity for A2451 was observed with denatured 23S rRNA (6). These observations suggest that proper folding of 23S rRNA must be achieved to confer peptidyl transferase activity.

The question of how this machinery developed remains of great interest. To investigate the possible evolutionary development of the peptidyl transfer reaction in a way different from done previously, we tried to obtain peptide bond formation without an exogenous ribozyme catalyst, using an aminoacyl-minihelix and an oligonucleotide model substrate containing Pm. The idea was to come up with the simplest possible system that used a minihelix-like motif as one of the participants, and see whether peptide bond formation was possible with such a simple system. In designing the model, we focused on the CCA sequence that is conserved among all tRNAs at the 3′ end of the minihelix domain (8). Chemical footprinting showed that the CCA regions of tRNA are in close contact with specific bases in 23S rRNA, several of which correspond to the sites of protection by antibiotics (9). G2252 in *E. coli* 23S rRNA has been proposed to interact with C74 by Watson–Crick base pairing (10), and the structure of the *H. marismortui* ribosome with bound CCdA-p-Pm showed a Watson–Crick interaction between C74 and G2252 (in *E. coli*) and C75 and G2251 (in *E. coli*) (5). Point mutations at any of the CCA nucleotides in tRNA abolished the peptidyl transfer activity (11). Thus, we made several model molecules based on these simple principles and tried to find conditions favorable for peptide bond formation, in the absence of the ribosome or of a catalytic RNA (ribozyme).

Materials and Methods

Synthesis of Minihelix^{Ala} and Pm-Containing Molecules. Minihelix^{Ala} was synthesized on an Expedite 8909 synthesizer (PE BioSystems, Foster City, CA). Pm containing deoxyribonucleotides (Pm-TGGT, Pm-TTGGT, Pm-TTGT, Pm-TTTGT, Pm-TTTT, Pm-TTTTT) also were synthesized on the same machine by using Pm-CPG, dG-5′-CE phosphoramidite, and dT-5′-CE phosphoramidite (Glen Research, Sterling, VA). Puromycin-RNA substrate (Pm-UGGU) was synthesized kindly by Dharmacon Research (Boulder, CO). Minihelix^{Ala} was deprotected by using the methods of Wincott *et al.* (12). For Pm-UGGU, 100 mM tetramethylethylenediamine-acetate (pH 3.8) was used for 2′-*O*-orthoester deprotection (Technical Bulletin 001; Dharmacon Research). All molecules were purified by using an ion exchange-HPLC column (DNAPac PA-100; Dionex).

Peptidyl Transfer Reaction Using *N*-acetyl-[^{14}C]alanyl-RNA^{Ala}. *N*-acetyl-[^{14}C]alanyl-minihelix^{Ala} was prepared according to Sardesai *et al.* (2). Ribosomes were prepared from *E. coli* Q13 (Hfr, RNase I$^-$, PNPase$^-$, *met*$^-$, *tyr*$^-$) according to a published procedure (13). The reaction mixture contained 50 mM Hepes·NaOH (pH 7.5), 1,000 mM NaCl, 10 mM $MgCl_2$, ≈20 μM *N*-acetyl-[^{14}C]alanyl-RNA^{Ala}, and ≈500 μM Pm-containing substrate. After the incubation at 0°C for 2 h, an equal volume of 20–2,000 mM imidazole solution (pH 8.3 with HCl) was added, and the sample tubes were put on ice for 7 days. The reaction mixture for the control reaction (usual fragment reaction; ref. 14) contained (before methanol addition) 50 mM Tris·HCl (pH 7.5), 0.4 M KCl, 20 mM $Mg(OAc)_2$, ≈3.0 A_{260} units of ribosomes, ≈500 μM Pm-containing substrate, ≈10 μM *N*-acetyl-[^{14}C]alanyl-RNA^{Ala}, and 33% methanol.

Abbreviation: Pm, puromycin.

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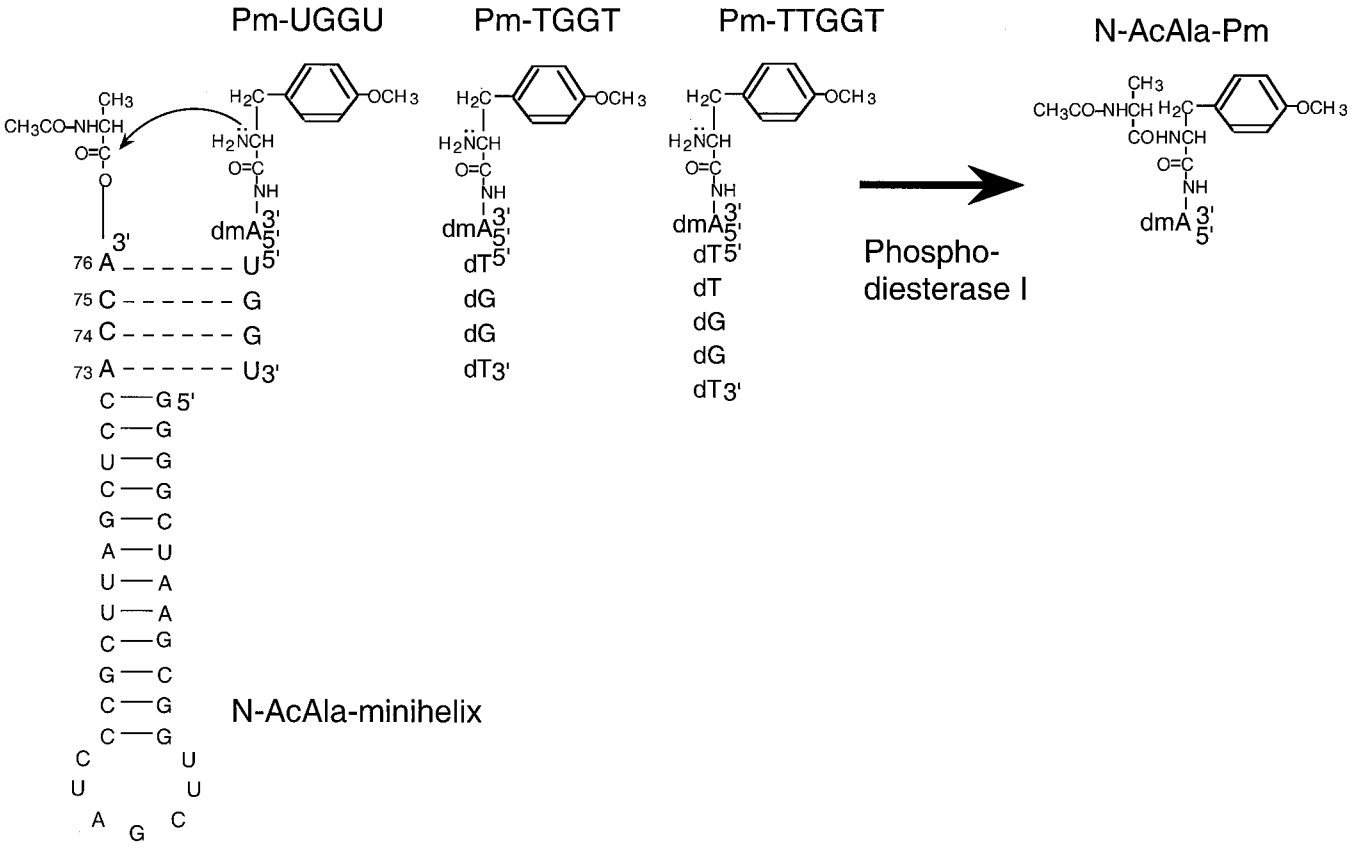

Fig. 1. Pm-containing oligonucleotides substrates and minihelix^{Ala} used in this study. dmA, *N*,*N*-dimethyladenosine.

TLC. The reaction product was treated with phosphodiesterase I (from bovine intestinal mucosa) to liberate any *N*-acetylalanyl-Pm that might form. In this reaction, imidazole seemed to interfere with the reaction, so the reaction mixture (before the treatment with phosphodiesterase I) was applied to a Ni-NTA agarose column for the removal of imidazole. Enzyme (1.5 units/ml) was added to the reaction mixture, and the incubation was performed at 37°C for 12 h. The products were resolved on TLC by using silica gel 60 F254 plates (EM Science). The products were eluted with chloroform/methanol/acetic acid [85:10:5 (vol/vol)]. The results were visualized on a PhosphorImager screen (LE177-906; Molecular Dynamics).

PAGE. Amino acid transfer from minihelix to Pm-DNA substrates were detected by denaturing 22% PAGE. After the electrophoresis, the gel was dried and subjected to visualization by phosphorimaging.

Mass Spectrometry. In these experiments, nonradioactive materials were used. The Sep-Pak C18 cartridge (Waters) was used to remove salts in the reaction mixture. The final product, *N*-acetylalanyl-Pm, was characterized by electron spray mass spectroscopy (API 100; MPE-Sciex, Alberta, Canada).

Results

Apparent Formation of Peptide Bond. The first model molecule (Pm-UGGU) used in this study has UGGU linked with Pm by a 5′–5′ phosphodiester bond (Fig. 1). UGGU was designed to base pair with the single-strand ACCA of *N*-acetylalanyl-minihelix (Fig. 1). Two uridines were added to the model molecule to strengthen its binding through base pairing with A73 and A76. The Pm-UGGU was incubated with *N*-acetylalanyl-minihelix in an attempt to form a peptide bond between the α-amino group of Pm and the carbonyl group of *N*-acetylalanine that is esterified to the 3′-terminal adenosine of the minihelix. The results of TLC analysis in the presence and absence of imidazole in the reaction mixture (after treatment with phosphodiesterase I to liberate any *N*-acetylalanyl-Pm that might form) resulted in a new species having the same mobility as *N*-acetylalanyl-Pm (Fig. 2*a*). (The *N*-acetylalanyl-Pm control was generated in a ribosome-catalyzed fragment reaction; ref. 14.) Formation of the putative peptide depended on the addition of imidazole.

Because the substrates were favorable in terms of synthesis, we also performed similar experiments with Pm-DNA substrates. These substrates (Pm-TGGT and Pm-TTGGT) linked TGGT or TTGGT with Pm by a 5′–5′ phosphodiester bond (Fig. 1). The result for Pm-TGGT (and Pm-TTGGT) was similar to that for Pm-UGGU (Fig. 2*b*). Once again, putative peptide bond formation depended on the presence of imidazole. For both Pm-TGGT and Pm-TTGGT, TLC analysis showed new spots having the same mobility as *N*-acetylalanyl-Pm (Fig. 3).

Mass spectrometry of the product after phosphodiesterase I digestion gave a peak at 585 Da, which is the expected mass of the protonated form of *N*-acetylalanyl-Pm (Fig. 4). We detected the peak at 585 Da with all three substrates, that is, Pm-UGGU, Pm-TGGT, and Pm-TTGGT. These results strongly confirm that the reaction produced a peptide bond in the absence of the ribosome.

Enhancement of Peptide Bond Formation by Imidazole. The results in Fig. 2 using TLC analysis showed that imidazole was required for peptide bond formation. To study further the effects of imidazole on peptide bond formation, both Pm-TTGGT and Pm-

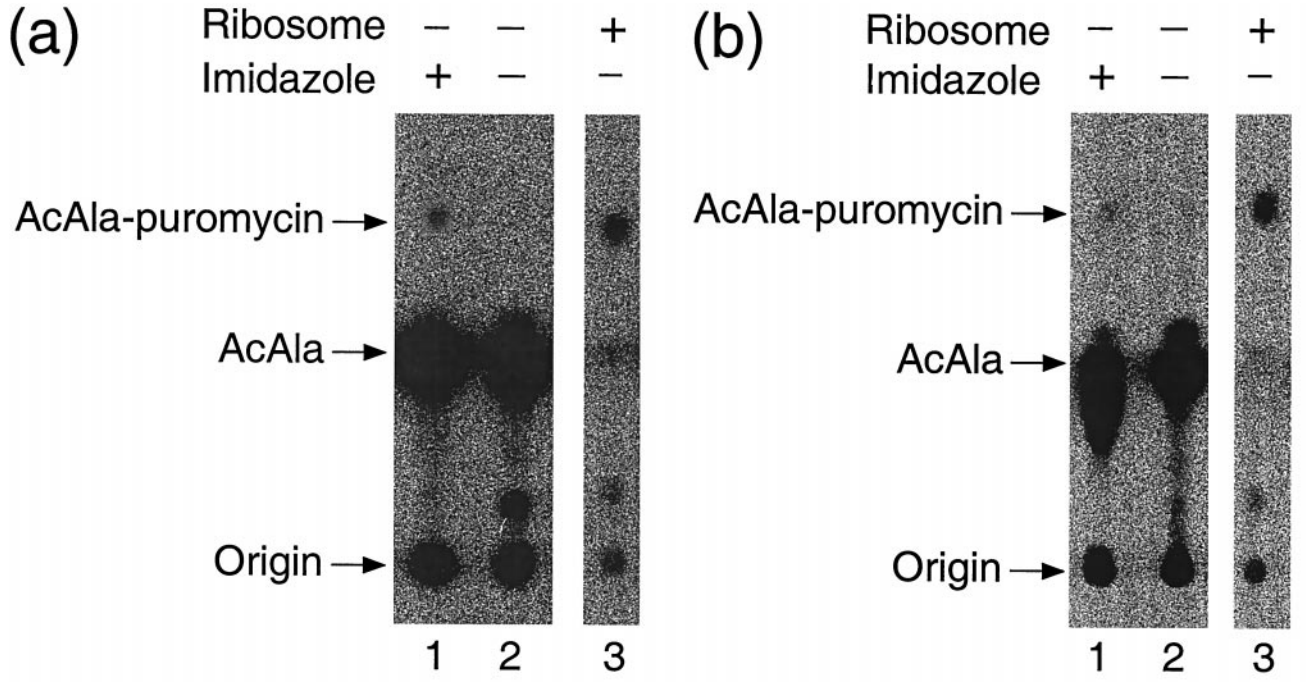

Fig. 2. (*a*) TLC analysis of reaction products produced from *N*-acetyl-[^{14}C]alanyl-minihelix and Pm-UGGU. Lane 1, in the presence of 1 M imidazole (without the ribosome); lane 2, in the absence of imidazole (without the ribosome); lane 3, in the presence of ribosome from *E. coli*. (*b*) TLC analysis of reaction products produced from *N*-acetyl-[^{14}C]alanyl-minihelix and Pm-TGGT. Lane 1, in the presence of 1 M imidazole (without the ribosome); lane 2, in the absence of imidazole (without the ribosome); lane 3, in the presence of ribosome from *E. coli*.

TGGT were used. Similar results were obtained with each. Because TLC analysis can give a misleading spot that has the same mobility as that of authentic product (7), for some experiments (such as these), we used gel electrophoresis in parallel with TLC analysis. The Pm-DNA substrates are much smaller than the minihelix, so that size differences in the DNA-containing piece could be detected on the gels when peptide synthesis occurred.

For the control with the usual fragment reaction (14), Pm-

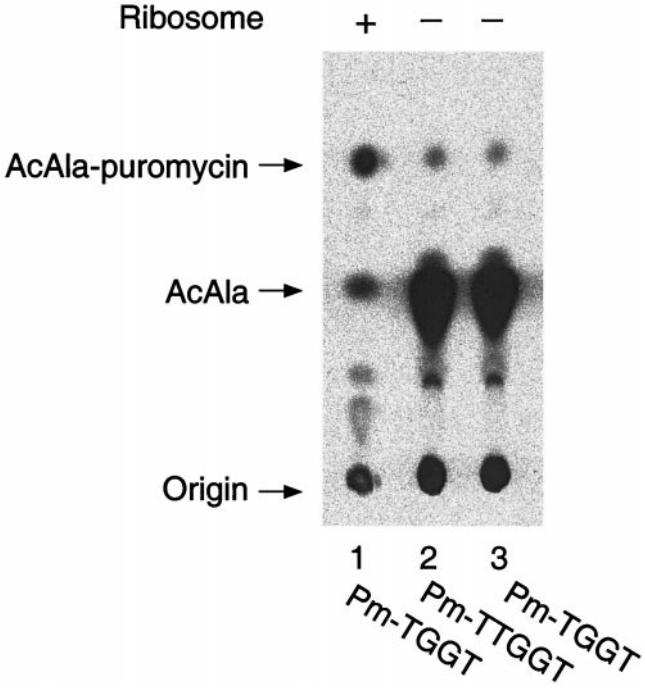

Fig. 3. TLC analysis of reaction products produced from *N*-acetyl-[^{14}C]alanyl-minihelix and the following Pm-DNA substrates. Lane 1, Pm-TGGT in the presence of ribosome from *E. coli*; lane 2, Pm-TTGGT in the presence of 1 M imidazole (without the ribosome); lane 3, Pm-TGGT in the presence of 1 M imidazole (without the ribosome).

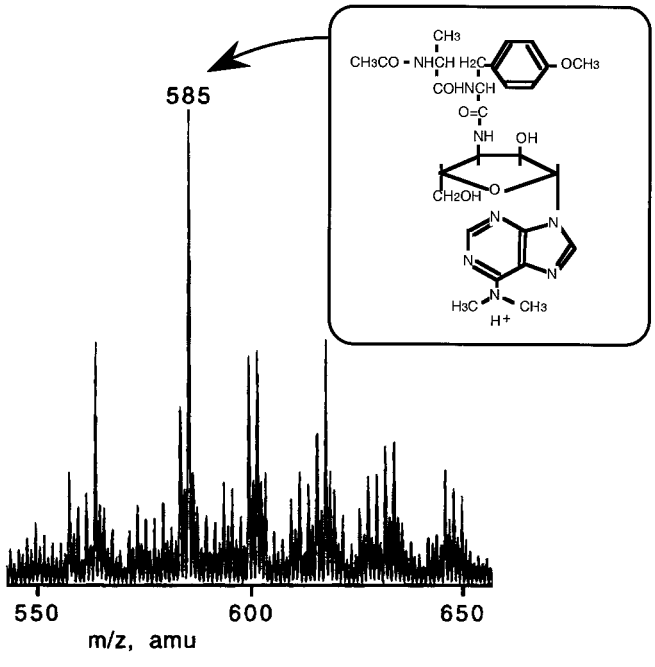

Fig. 4. Mass spectrometry of reaction products produced from *N*-acetylalanyl-minihelix and Pm-TGGT in the presence of imidazole (without the ribosome). The peak at 585 corresponds to the protonated form of *N*-acetylalanyl-Pm.

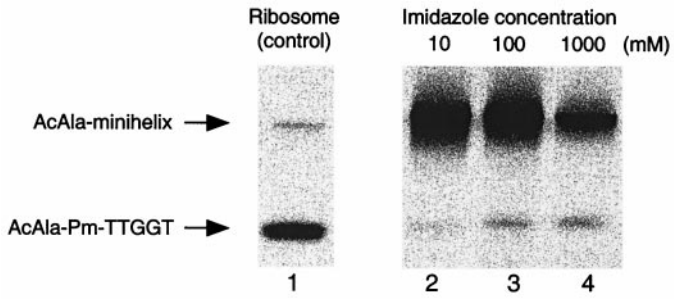

Fig. 5. Gel electrophoresis of reaction products produced from *N*-acetyl-[^{14}C]alanyl-minihelix and Pm-TTGGT. Lane 1, in the presence of ribosome from *E. coli*; lane 2, in the presence of 10 mM imidazole (without the ribosome); lane 3, in the presence of 100 mM imidazole (without the ribosome); lane 4, in the presence of 1 M imidazole (without the ribosome).

TTGGT was the substrate in the presence of the ribosome and minihelix, and in the absence of imidazole. Bands were detected not only at the position of *N*-acetylalanyl-minihelix, but also at the position of *N*-acetylalanyl-Pm-TTGGT (Fig. 5). Although no reaction was seen in the absence of imidazole (Fig. 2), peptide was produced without the ribosome but in the presence of imidazole, with the amount of peptide increasing when the concentration of imidazole was increased from 10 to 100 mM (Fig. 5). At 100 mM imidazole (Fig. 5, lane 3), we estimate the ratio of product *N*-acetylalanyl-Pm-TTGGT to *N*-acetylalanyl-minihelix to be 0.02, to give an approximate yield of 2%. [Raising further the concentration of imidazole resulted in some deacylation of the *N*-acetylalanyl-minihelix (Fig. 5, lane 4), so that the yield of peptide was not enhanced beyond that seen at 100 mM imidazole.] Thus, imidazole is a catalyst for peptide bond formation.

Effect of Base Pairing on Peptide Bond Formation. To investigate the effect of base pairing with the CCA trinucleotide, we used "mutated" substrates and Pm itself for peptide bond formation. The normal substrate (Pm-TTGGT) showed a band not only at the position of *N*-acetylalanyl-minihelix but also at the position of *N*-acetylalanyl-Pm-TTGGT (Fig. 6). (Similar results were obtained for Pm-TGGT; data not shown.) In contrast, in the case of both Pm-TTTGT (which has a mismatch with C75) and Pm-TTTTT (which has mismatches with both C74 and C75), a much fainter band was detected at the position of *N*-acetylalanyl-Pm-TTTGT and -Pm-TTTTT, respectively (Fig. 6). In the case of the negative control, Pm, no bands were detected at any position other than what is associated with *N*-acetylalanyl-minihelix. These results demonstrate the importance of close proximity (caused by the base pairing interaction) for production of a peptide.

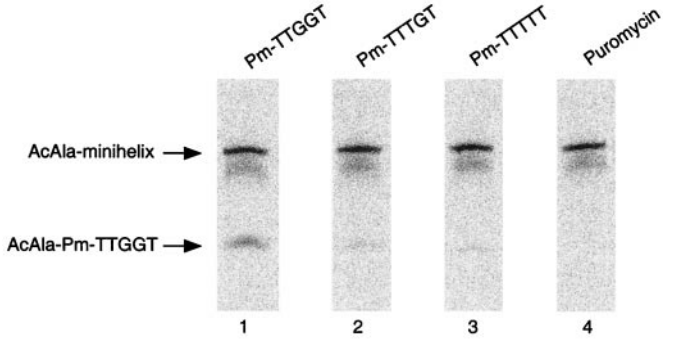

Fig. 6. Gel electrophoresis of reaction products produced from *N*-acetyl-[^{14}C]alanyl-minihelix and the following substrates (without the ribosome). Lane 1, Pm-TTGGT in the presence of 1 M imidazole; lane 2, Pm-TTTGT in the presence of 1 M imidazole; lane 3, Pm-TTTTT in the presence of 1 M imidazole; lane 4, Pm in the presence of 1 M imidazole.

Discussion

Early work by Weber and Orgel (15) showed the importance of proximity effects in generating peptide bonds from activated amino acids. In that work, aminoacyl esters of derivatives of adenosine were tethered onto a polyuridylic acid template to generate cyclic diketopiperazine products. [Synthesis of the linear Gly-Gly dipeptide was not observed, because the nascent-activated dipeptide cyclyzed to the diketopiperazine. Because an imidazole buffer (250 mM) was used, and in view of the importance of imidazole in the work reported here, it is likely that imidazole catalysis was also critical in at least the initial production of activated dipeptide.] The product of the concentrations of poly U and aminoacyl derivatives was about 4 orders of magnitude higher than the product of concentrations of nucleic acid species used here. This large difference is presumably due, at least in part, to the poly U-directed hydrogen-bonded complexes involving one or two base pairs being weaker than the multiple base pairs used for complex formation in this work. However, had oligoadenylic acid species with three or more monomer units been used to direct binding to poly U, it is likely that the spacing of poly U-bound activated glycine (attached to oligo A) would have been too great to achieve peptide synthesis. Thus, by using simple mono- and dinucleotide derivatives as carriers of activated glycine, proximity could be achieved. At the same time, the restriction on the size of the oligomers made inherently difficult the generation of more stable reactive complexes.

Because our oligonucleotide-based system took advantage of the common CCA trinucleotide of tRNAs, and because this trinucleotide appears critical to tether tRNA at the peptidyl transferase center of the 50S particle, the possibility that a version of the ribosome-free system described here was the progenitor of modern peptide biosynthesis has to be considered. One of our oligonucleotides was based on the minihelix domain of tRNA. Because the minihelix by itself is known to interact with the peptidyl transferase center on the ribosome (2), and because the transition from minihelix to full tRNA with its template reading head is conceptually straightforward, the system we used can easily be imagined to evolve into something that more closely resembles the one based on the ribosome. A significant difference between what was used here and the ribosome-based system is in the details of how the CCA trinucleotide is used. In this work, two aminoacylated oligonucleotides interact directly through base complementarity with the CCA triplet of the minihelix. In the ribosome, the CCA triplet of the minihelix domain of tRNA interacts instead with noncontiguous bases in 23S rRNA (9, 10). This interaction helps to tether tRNA at the peptidyl transferase center and facilitates steric proximity of the reacting peptidyl- and aminoacyl-tRNA species. Thus, if the simple ribosome-free systems used here was to progress, the next step in its development would be introduction of an RNA species that hybridized to one or both of the reactive oligonucleotides through the CCA sequence, and achieved proximity of the reactive species without the necessity of a 5′–5′ phosphate linkage in one of the substrates (Fig. 1). This RNA species might also enhance peptidyl transferase activity by acting as a ribozyme. In fact, because the yield in our system is roughly 2% (see above), so that considerable improvement could in principle be achieved by having a specific RNA catalyst.

Recently, *in vitro* selection experiments showed the possibility that RNA can catalyze peptide bond formation. CCdA-p-Pm-binding aptamers were developed that contain an eight-base binding motif matching the peptidyl-transferase loop (A2448–

G2455) of 23S rRNA (16). Peptidyl-transferase catalytic RNA was also selected to use an AMP-Met-biotin substrate. This ribozyme had some similarities in its RNA structure to a CCdA-p-Pm binding aptamer developed by Welch *et al.* (17, 18). However, the product yields obtained were very low compared with that achieved by ribosome. In the present study, a direct interaction between two oligonucleotides was sufficient to obtain peptide bond formation without the aid of a ribozyme. The finding that the mismatched Pm-TTTGT and Pm-TTTTT oligonucleotides produced little peptide bond is consistent with this conclusion.

Although these results emphasize the significance of proximity effects for peptide bond formation, the data in Fig. 2 (and also Fig. 5) show that proximity alone is not sufficient. In addition, a catalyst is required. In these studies, imidazole serves as a catalyst presumably because the lone pair of a tertiary nitrogen can abstract a proton from the α-amino group of Pm. The carbonyl carbon of *N*-acetylalanyl-minihelix then undergoes nucleophilic attack by the deprotonated amino group. In the cocrystal structure of the large ribosomal subunit and CCdA-p-Pm, only N3 of A2486 has been identified as a candidate for catalysis of the peptidyl transfer reaction (5). The pKa of the N1 or N3 of A2451 in *E. coli* was estimated to be around neutrality (6), similar to that of free imidazole.

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