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LETTER TO THE EDITOR

Peptidyl transferase activity catalyzed by protein-free 23S ribosomal RNA remains elusive

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In two recent articles by Nitta et al. (1998a, 1998b), evidence was presented that in vitro-transcribed protein-free 23S rRNA of *Escherichia coli* (and its individual domains) can catalyze peptide bond formation. The implications of these studies are considerable because demonstration of catalytic activity associated with protein-free 23S rRNA would be fundamental to our understanding of the mechanism and evolution of protein synthesis (Noller et al., 1992; Khaitovich et al., 1999).

In our three laboratories, we attempted to reproduce the key experiment of Nitta et al. (1998a, 1998b) where peptide bond formation was catalyzed by isolated 23S rRNA. We followed as closely as possible all of the described reaction conditions (Nitta et al., 1998a, 1998b). The assay used by Nitta et al. (1998a, 1998b) ostensibly followed the formation of a dipeptide, acetyl diphenylalanine (AcPhe-Phe), from the peptidyl-tRNA, AcPhe-tRNA, and the aminoacyl-tRNA, Phe-tRNA. However, in neither of the published papers were these two substrates incubated together with the intact 70S ribosome (and polyU) as a positive control to show ribosome-catalyzed formation of authentic AcPhe-Phe. In our experiments, when radiolabeled AcPhe- and Phe-tRNAs were incubated with 70S ribosomes (an equimolar mixture of 50S + 30S ribosomal subunits) and poly U and the products were analyzed in the TLC system described (Nitta et al., 1998b), two main radioactive products appeared (Fig. 1A, lane 3). The reaction products were recovered from the TLC plate and identified as acetyl diphenylalanine and acetyl triphenylalanine (AcPhe-Phe and AcPhe-Phe-Phe) by mass spectrometry (molecular weight 355 for AcPhe-Phe and 502 for AcPhe-Phe-Phe; data not shown). These are the anticipated products of an authentic ribosome-catalyzed peptidyl transferase reaction (Monro et al., 1969); thus, the substrates prepared according to the protocols described by Nitta et al. (1998b) were active. This ribosomal reaction provides a reliable marker for the dipeptidyl product, AcPhe-Phe.

Next, when these substrates (AcPhe-tRNA and Phe-tRNA) were incubated with in vitro-transcribed 23S rRNA, a reaction product X was formed (Fig. 1A, lane 4) that had similar, though not identical (compare lane 3 with lanes 4 and 5 in Fig. 1A and lane 7 with lane 8 in Fig. 1B), migration to the radioactive product identified as AcPhe-Phe in Figure 1A. These results mirror those reported in Nitta et al. (1998a, 1998b). Remarkably, a radioactive product X with identical migration was formed when these substrates were incubated with 16S rRNA (Fig. 1A, lane 5), with group I intron RNA or with RNA run-off transcripts generated from *Xmn*1 and *Alw*N1 digested pUC18 plasmid; there is no expectation that these RNAs will catalyze peptidyl transferase. In light of these results, we discovered that in the absence of incubation with any RNA, the same radioactive product X was produced from these tRNA substrates in the presence of low concentrations of ethanol (Fig. 1B, lanes 9/10 and Fig. 1C, lanes 14/15).

Preparation of in vitro-transcribed RNA involves an ethanol precipitation as the final step (Nitta et al., 1998a, 1998b). Appearance of the product X in the RNA-only samples depended critically on the amount of ethanol in RNA preparations (Fig. 2). When, after precipitation, ethanol was simply aspirated from the 23S rRNA or 16S rRNA pellets, prior to addition of incubation buffer,

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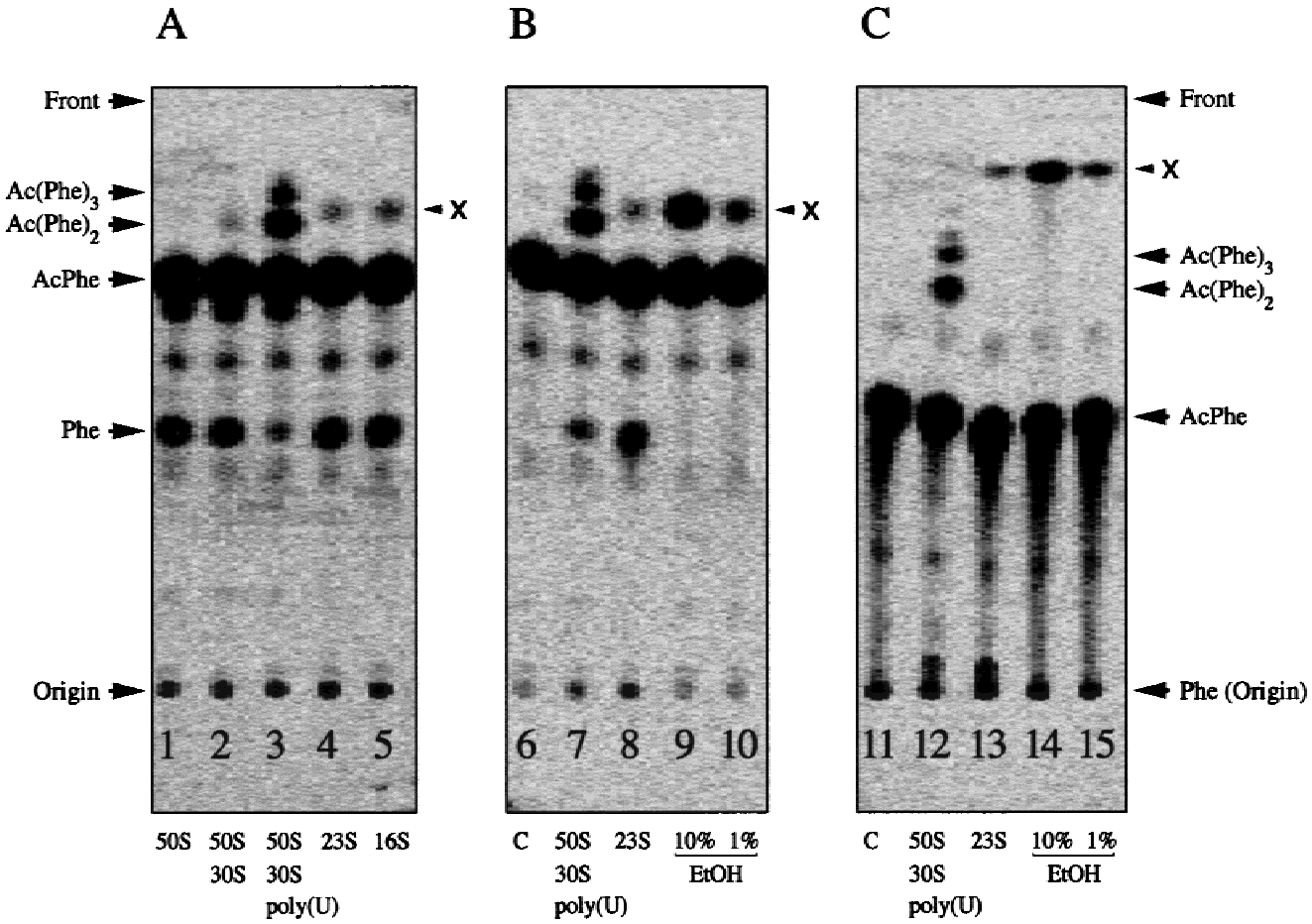

FIGURE 1. TLC analysis of reaction products produced from Ac-[^{14}C]-Phe-tRNA and [^{14}C]-Phe-tRNA in the presence of ribosomal subunits, in vitro transcribed rRNA, or ethanol. The substrates were incubated under experimental conditions described in Nitta et al. (1998b) in the presence of: lane 1: isolated 50S ribosomal subunits from *E. coli*; lane 2: equimolar mixture of 30S and 50S ribosomal subunits from *E. coli*; lanes 3, 7, and 12: equimolar mixture of 30S and 50S ribosomal subunits plus 25 μg poly (U); lanes 4, 8, and 13: in vitro-transcribed 23S rRNA; lane 5: in vitro-transcribed 16S rRNA; lanes 6 and 11: AcPhe-tRNA incubated under the above conditions without addition of other ingredients; lanes 9 and 14: same as lanes 6 and 11, but in the presence of 10% ethanol; lanes 10 and 15: same as lanes 6 and 11, but in the presence of 1% ethanol. Reaction products were loaded onto silica TLC plates (Merck) and chromatographed in (**A**,**B**) *n*-butanol:acetic acid:water (4:0.9:1) [this system was used extensively in both articles by Nitta et al. (1998a, 1998b)] or (**C**) chloroform: methanol:acetic acid (85:10:5). Product markers (Phe, AcPhe, Ac(Phe)$_2$ and Ac(Phe)$_3$) are indicated by arrows. The spot corresponding to the product that appears in the reaction in the presence of in vitro-transcribed RNA (or ethanol) is designated by X. The sample loading origin (Origin) and the solvent front (Front) are indicated. **Experimental**: The substrates, Ac-[^{14}C]-Phe-tRNA and [^{14}C]-Phe-tRNA, were synthesized according to Moazed & Noller (1989) and Rappoport & Lapidot (1974), and HPLC-purified according to Nitta et al. (1998b). Ribosomal subunits were isolated following standard protocols (Spedding, 1990). In vitro-transcribed 23S and 16S rRNA were prepared following as closely as possible the protocols described in Nitta et al. (1998a, 1998b), using minor modifications provided by Dr. Nitta (pers. comm.). The standard reaction mixture (total volume 25 μL) contained 2 μM 23S rRNA or 0.3 μM of ribosomal subunits, 2 μM Ac-[^{14}C]-Phe-tRNA, 0.1 μM [^{14}C]-Phe-tRNA, 50 mM HEPES-KOH (pH 8.2), 30 mM $MgCl_2$, and 160 mM NH_4Cl. Incubation conditions and processing of the samples were according to Nitta et al. (1998b).

the reaction mixture resulted in formation of product X (Fig. 2, lanes 2 and 5). In contrast, when the RNA (16S or 23S) was extensively dried after ethanol precipitation, no product formation was observed (Fig. 2, lanes 3 and 6). Addition of ethanol back to the "over-dried" RNA samples restored appearance of the product spot X (Fig. 2, lane 4). In the absence of added RNA, a spot of the same intensity as the one observed in the presence of 23S rRNA (Fig. 1B, lane 8) was obtained by incubation of reaction substrates in the presence of ~0.3% ethanol, a concentration consistent with the levels of residual ethanol expected in preparations of 23S and 16S rRNA.

On further examination, we observed that the product X of the RNA-only catalyzed reaction could be resolved from the authentic AcPhe-Phe product in an alternative TLC system composed of chloroform:methanol:acetic acid (85:10:5) (Fig. 1C). In this system, the

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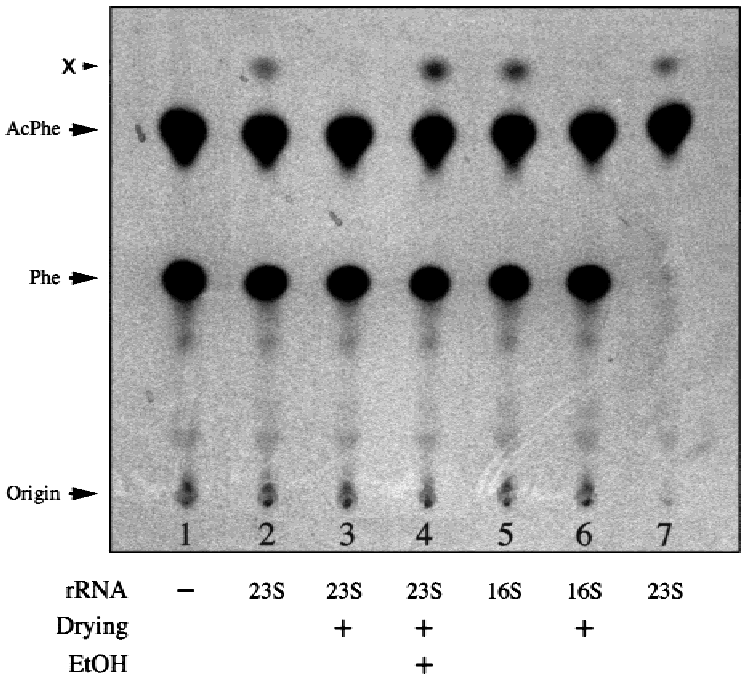

FIGURE 2. TLC analysis of reaction products produced from Ac-[^{14}C]-Phe-tRNA and [^{14}C]-Phe-tRNA in the presence of differently treated in vitro-transcribed rRNA. The substrates were incubated under experimental conditions described in Nitta et al. (1998a) in the presence of: lane 1: no RNA; lane 2: in vitro-transcribed 23S rRNA precipitated with ethanol directly prior to resuspension in the reaction mixture; lane 3: in vitro-transcribed 23S rRNA precipitated with ethanol, dried extensively in a speed-vac, and resuspended in the reaction mixture; lane 4: in vitro-transcribed 23S rRNA precipitated with ethanol, dried extensively in a speed-vac, and resuspended in the reaction mixture with 10% ethanol added; lane 5: in vitro-transcribed 16S rRNA precipitated with ethanol directly prior to resuspension in the reaction mixture; lane 6: in vitro-transcribed 16S rRNA precipitated with ethanol, dried extensively in a speed-vac, and resuspended in the reaction mixture; lane 7: in vitro-transcribed 23S rRNA precipitated with ethanol directly prior to resuspension in the reaction mixture, incubated only with Ac-[^{14}C]-Phe-tRNA. **Experimental**: The substrates, Ac-[^{14}C]-Phe-tRNA and [^{14}C]-Phe-tRNA, were synthesized according to Moazed & Noller (1989) and Rappoport & Lapidot (1974). In vitro transcribed 23S and 16S rRNA were prepared according to Green & Noller (1996); resuspension of RNAs following G50 purification was in "self-folding" buffer (Nitta et al., 1998a). The standard reaction mixture (total volume 20 μL) contained 100 μg of in vitro-transcribed RNA, 3 μM Ac-[^{14}C]-Phe-tRNA, 3 μM [^{14}C]-Phe-tRNA, 50 mM HEPES-KOH (pH 8.2), 100 mM $MgCl_2$, and 0.5% SDS. Incubation conditions and subsequent processing of the samples were according to Nitta et al. (1998a).

mobility of the radioactive product X was substantially different from the mobility of the AcPhe-Phe standard. No radioactivity was detected at the position corresponding to AcPhe-Phe in the absence of intact ribosomes (Fig. 1C, lane 13). The ethanol product comigrated on silica plates with Ac-Phe ethyl ester marker (obtained from Sigma) in three different chromatographic systems [chloroform:methanol (5:1), chloroform:methanol: acetic acid (85:10:5) and *n*-butanol:acetic acid:water (13:4:5)]. The resolution of thin layer chromatography is maximal at Rf values of ~0.3–0.5; the putative dipeptide product reported in Nitta et al. (1998a, 1998b) had an Rf of >0.8 in their TLC system composed of *n*-butanol:acetic acid:H_2O (4:0.9:1). When the radioactive product X was isolated from the TLC and treated with alkali, radioactive Ac-Phe was produced (Fahnestock et al., 1970), compatible with the ester nature of the product. Thus we conclude that the product X formed in our reaction tubes containing Ac-Phe-tRNA, Phe-tRNA, and conventionally prepared 23S rRNA (or other RNAs) is the ethanolysis product of Ac-Phe-tRNA. Final confirmation of this prediction was demonstrated by the fact that the reaction product X did not depend on the presence of Phe-tRNA, but only on the presence of Ac-Phe-tRNA (Fig. 2, lane 7).

The controversial nature of the data presented by Nitta et al. (1998a, 1998b) stems from the lack of reliable AcPhe-Phe standards used by the authors. While in the original paper (Nitta et al., 1998a) no ribosomal standard was used,[4] in the more recent paper (Nitta et al., 1998b), the authors used as a standard the product formed in high yield from AcPhe-tRNA and Phe-tRNA in the presence of 50S ribosomal subunits (Fig. 1, lane 4, in Nitta et al., 1998b). However, it previously has been documented that isolated 50S ribosomal subunits do not catalyze formation of dipeptide from these substrates unless significant amounts of ethanol or methanol are included in the reaction (Monro, 1969; Monro et al., 1969) (Fig. 1A, lane 1). Indeed, small amounts of AcPhe-Phe are formed even in the reaction catalyzed by 70S ribosomes unless poly (U) is included (Fig. 1A, lanes 2 and 3; Rheinberger et al., 1981).

Our data do not rule out the possibility of catalysis by protein-free 23S rRNA. Here, it is merely reported that we were unable to catalyze the formation of authentic AcPhe-Phe dipeptidyl product using naked 23S rRNA as recently reported by Nitta et al. (1998a, 1998b). The apparent discrepancy between our findings and those of Nitta et al. (1998a, 1998b) may be explained by alcohol contamination in RNA preparations resulting in the production of Ac-Phe-tRNA esters.

[4] In the original paper (Nitta et al., 1998a; legend to Fig. 2), the authors describe the use of synthetic AcPhe and AcPhe-Phe prepared by acetylation of Phe or Phe-Phe by incubation with sodium carbonate. Because treatment of amino acids (or dipeptides) with sodium carbonate will not result in acetylation, we have assumed that this error results from a misprint in the manuscript or incorrect procedure.

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