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Specific Inactivation of *Escherichia coli* $tRNA^{Phe}$ by Antisense DNA-Treatment Under Mg^{2+}-Deficient Conditions

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Abstract—The preparation of an *Escherichia coli* tRNA mixture lacking several specific species may be useful for applications ranging from cell-free protein preparation to protein engineering. We have already demonstrated that $tRNA^{Asp}$ can be inactivated, or 'knocked out', with practical specificity by an antisense strategy. In the present study, we synthesized five $tRNA^{Phe}$-targeted antisense oligonucleotides and tested if this tRNA can also be inactivated specifically. The salt conditions used previously for the $tRNA^{Asp}$ inactivation were not applicable to $tRNA^{Phe}$. Instead, Mg^{2+}-deficient conditions were found to be useful for the inactivation of $tRNA^{Phe}$ by the antisense oligonucleotides. These conditions were also applicable to the inactivation of $tRNA^{Asp}$. The susceptibility to the antisense DNAs can change drastically, depending on the concentration of Mg^{2+}.

Introduction

Cell-free protein synthesis, driven by crude S30 extracts from *Escherichia coli*, has been applied to prepare proteins containing unnatural amino acids at specific positions. This technique has been used for detailed mutagenic analyses of protein structure–function relationships,[1,2] analyses of the mitochondrial protein import pathway,[3,4] and studies of electron transfer on the surface of the modified proteins.[5] Specific stable-isotope labeling of proteins for efficient NMR signal assignments was also achieved by using an enzymatically aminoacylated tRNA that read the amber stop codon.[6,7]

In these applications, the full-length protein products can be separated from the prematurely terminated by-products by their molecular sizes. In many cases, a tRNA molecule charged with the amino acid was designed to suppress a stop codon placed at the intended position on the mRNA. This stop-codon dependent system for the introduction of unnatural/labeled amino acids (stop codon system) is convenient because the read-through product indisputably has the unnatural/labeled amino acid at the intended position.[1–4,6,7] In the other cases, a +1 frameshift was induced by an unnatural aminoacyl-tRNA with a four-base anticodon, which also certifies that the full-length product has the amino acid at the frameshift position (four-base codon system).[5]

However, these strategies sacrifice the possibility of multiple introductions of unnatural/labeled amino acid residues in a single polypeptide chain. The aminoacyl-tRNA competes, at each site, with another component of the S30-based translation reaction. In the case of the stop codon system, the peptide chain release factor terminates the synthesis. In the case of the four-base codon system, the intrinsic natural aminoacyl-tRNA(s) insert(s) the natural amino acid.

On the other hand, some cell-free translation systems used for studies of the kinetic aspects of protein synthesis[8] are totally dependent on the addition of tRNA molecules. In these systems, it is possible to reconstitute a cell-free translation with only a limited set of tRNA species. Thus, the artificial aminoacyl-tRNAs that read sense codons can be used, in principle, without any competing molecule. For the purpose of preparing proteins for further studies, it is necessary to prepare a tRNA mixture that can read most of the 61 sense codons but cannot read several specific codons. Therefore, we showed, in a previous study, that an *E. coli* tRNA mixture that lacks only a single tRNA species, $tRNA^{Asp}$, can be 'knocked out' by an antisense

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method.[9] The 'knock out' tRNA could be used in a cell-free translation system. If two different tRNA species can be inactivated by the method, then the simultaneous introduction of two amino acids into multiple, different sites of a single polypeptide may be possible. However, the melting profiles may be different from one tRNA species to another. Therefore, in the present study, we tested if another tRNA species, $tRNA^{Phe}$, can also be inactivated with antisense methods. We found that the susceptibility to the antisense DNAs can change drastically, depending on the concentration of Mg^{2+}.

Results and Discussion

Treatment of the tRNA mixture with $tRNA^{Phe}$-targeted antisense DNAs in the presence of Mg^{2+}

In the previous study, we designed five different antisense DNAs against $tRNA^{Asp}$ (Asp-1 to Asp-5; Table 1) and found that two of them can inactivate the tRNA with practical specificity, when incubated at 80 °C with an *E. coli* crude tRNA mixture and then gradually cooled to room temperature. Thus, we first synthesized the five oligonucleotides, listed in Table 1, targeted to the similar positions in $tRNA^{Phe}$ (Phe-1 to Phe-5) as in the case of the $tRNA^{Asp}$ inactivation. These $tRNA^{Phe}$-targeted oligonucleotides were evaluated in the same manner as in the previous study (Fig. 1). A solution of *E. coli* crude tRNA and one of the $tRNA^{Phe}$-targeted oligomers was incubated at 80 °C in the presence of RNase H and was cooled to room temperature. In this case, the solution contained 1 mM Mg^{2+}, which we initially expected to stabilize the antisense DNA–tRNA complex and to activate RNase H. However, none of these five oligonucleotides had both high efficiency and specificity in the inactivation of $tRNA^{Phe}$. Phe-1, 2, 3, and 4 had only small effects on the Phe-acceptor activity (filled squares, circles, triangles, and diamonds, respectively). Phe-5 blocked the acceptor activity only partially at 1–10 μM (see below) and caused nonspecific inhibition of Asp-acceptor activity at 64 μM (open squares). Thus, this salt condition may be unsuitable for these antisense oligonucleotides to block specific aminoacylation.

Table 1. Sequences of antisense oligodeoxyribonucleotides[a]

Name	Sequence	Target[b]
$tRNA^{Phe}$-targeted oligonucleotides		
Phe-1	5′-d(TACCGACTGAGCTATCCGGGC)-3′	1–21
Phe-2	5′-d(TTTTCAATCCCCTGCTCTACCGAC)-3′	14–38
Phe-3	5′-d(GACACGGGGATTTTCAATCCCCT)-3′	26–48
Phe-4	5′-d(ACCAAGGACACGGGGATTTTCAA)-3′	32–54
Phe-5	5′-d(TGGTGCCCGGACTCGGAATCGAA)-3′	54–76
$tRNA^{Asp}$-targeted oligonucleotides[c]		
Asp-1	5′-d(TAACCGACTGAACTACCGCTCC)-3′	1–21
Asp-2	5′-d(GTGACAGGCAGGTATTCTAACCGACT)-3′	14–38
Asp-3	5′-d(GACCCCCTGCGTGACAGGCAGGT)-3′	26–48
Asp-4	5′-d(ACCCGCGACCCCCTGCGTGACAG)-3′	32–54
Asp-5	5′-d(TGGCGGAACGGACGGGACTCGAA)-3′	54–76

[a]All oligonucleotides are unmodified, phosphodiester DNAs.
[b]tRNA portions that are complementary to the antisense oligonucleotides are shown by the residue numbers.
[c]$tRNA^{Asp}$-targeted oligonucleotides are the same as those used in the previous study.

Effects of Mg^{2+} on the efficacy of Phe-5

The NCp7 peptide from HIV-1 promotes annealing between the primer binding site of the HIV-1 genome and the 3′-portion of the primer $tRNA^{Lys-3}$.[10,11] This requires, in vitro, the presence of Zn^{2+} and a low-Mg^{2+} concentration. Thirteen out of the 14 positions in the acceptor stem of $tRNA^{Phe}$ are identical to those in $tRNA^{Lys-3}$,[12,13] and the structure of $tRNA^{Phe}$ is stabilized by the coordination of Mg^{2+} ions at specific sites.[14] Thus, we speculated that the Zn^{2+}-containing,

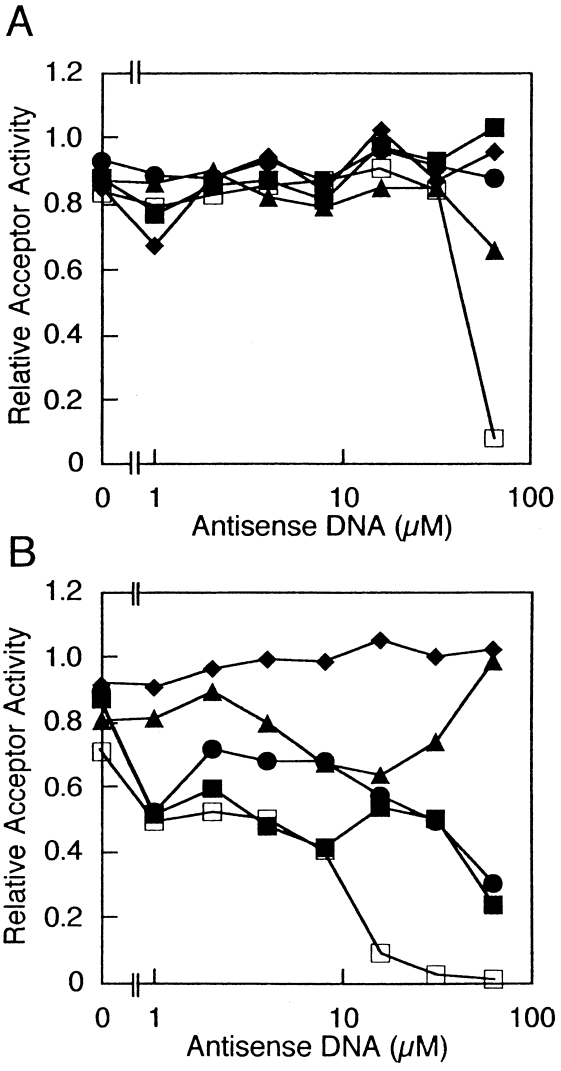

Figure 1. Effects of the treatment of the *Escherichia coli* tRNA mixture in the Mg^{2+}-containing solution with the $tRNA^{Phe}$-targeted antisense oligonucleotides on the acceptor activities of Asp (A) and Phe (B). Each antisense oligonucleotide was mixed with unfractionated *E. coli* tRNA (20 μg) in a solution (42 μL) containing 2 mM Tris–HCl, pH 7.4, 5 mM NaCl, 1 mM $MgCl_2$, and 0.17 unit/μL *Tth* RNase H. This solution was incubated at 80 °C for 5 min and was gradually cooled to room temperature. The activities are shown as the values relative to the activity of the same mixture without the antisense treatments. ■ Phe-1; ● Phe-2; ▲ Phe-3; ◆ Phe-4; □, Phe-5.

low-Mg^{2+} condition (in the absence of NCp7) may be suitable for the treatment by Phe-5, which targets to the 3′-portion of $tRNA^{Phe}$. Therefore, we examined the dependence of the Phe-acceptance on the concentration of Mg^{2+} during the treatment with Phe-5 (Fig. 2). Below a 0.1 mM Mg^{2+} concentation, Phe-5 efficiently inactivated $tRNA^{Phe}$ (circles), without affecting the Asp-acceptor activity (squares). Zn^{2+} had no effects on the Phe-acceptance (data not shown). In this experiment, Phe-5 (7.9 μM) did not block the Phe acceptance in the presence of more than 0.4 mM of Mg^{2+} ions, although it partially did in the experiment shown in Figure 1. This difference may be an effect of the difference in the concentrations of NaCl and/or Tris–HCl.

Specific inactivation of $tRNA^{Phe}$ in the absence of Mg^{2+}

We tested the effects of the five oligonucleotides under another Mg^{2+}-deficient condition, which is the same as what was suitable for the $tRNA^{Asp}$-targeted antisense DNAs, except for the concentration of Mg^{2+} (Fig. 3). Phe-1 (filled squares) and Phe-5 (open squares) effectively impaired the Phe-acceptor activity of the *E. coli* tRNA mixture at the concentration of 4 μM (Fig. 3A), without affecting the Asp-acceptor activity (Fig. 3B). Phe-2 also had some effect at higher concentrations (filled circles). Phe-3 (filled triangles) and Phe-4 (filled diamonds) were not effective under these conditions. Since Phe-1 and Phe-5 target the 5′- and 3′-portions, respectively, of $tRNA^{Phe}$, the acceptor stem may be more susceptible to the antisense oligonucleotides than the other stems in this tRNA under the Mg^{2+}-deficient conditions. The function of NCp7 in HIV-1 primer annealing may be related to the coordination of a Mg^{2+} ion, if any, to the acceptor stem of $tRNA^{Lys-3}$.

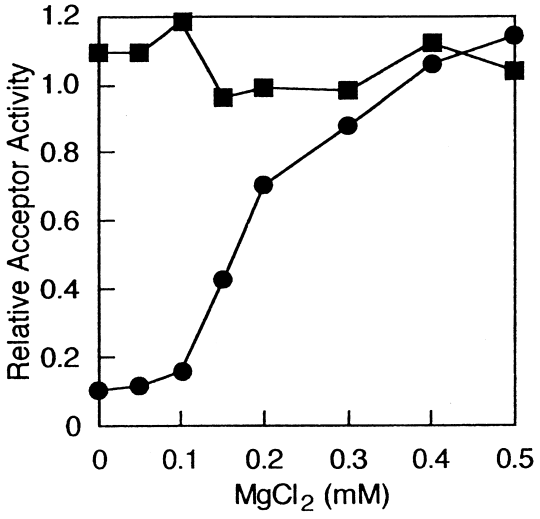

Figure 2. Mg^{2+}-dependence of the Asp- and Phe-acceptance of the Phe-5-treated tRNA mixtures. Phe-5 (7.9 μM) and the *Escherichia coli* tRNA mixture (20 μg) were incubated and cooled, as in Figure 1, in a buffer (42 μL) containing 30 mM Tris–HCl, pH 7.5, 30 mM NaCl, 10 μM $ZnCl_2$, 5 mM DTT, 0.17 unit/μL *Tth* RNase H, and $MgCl_2$. The activities are shown as the values relative to the activity of the same mixture without the antisense DNA treatments. ■ Asp-acceptor activity; ● Phe-acceptor activity.

Specific inactivation of $tRNA^{Asp}$ in the absence of Mg^{2+}

Many tRNAs coordinate Mg^{2+} ions, which may stabilize the secondary and tertiary structures.[15–20] Thus, we tested if $tRNA^{Asp}$ can also be inactivated under Mg^{2+}-deficient conditions (Fig. 4). All of the antisense DNAs were effective at the 8 μM Mg^{2+} concentration. Asp-5 was the most efficient, with activity at 1 μM. None of these antisense DNAs affected the Phe-acceptor activity. In the previous study, we showed that Asp-3 and Asp-4 are effective, and the other three are less effective, under the Mg^{2+}-containing condition. Thus, the melting profiles of $tRNA^{Asp}$ under these conditions may differ from each other.

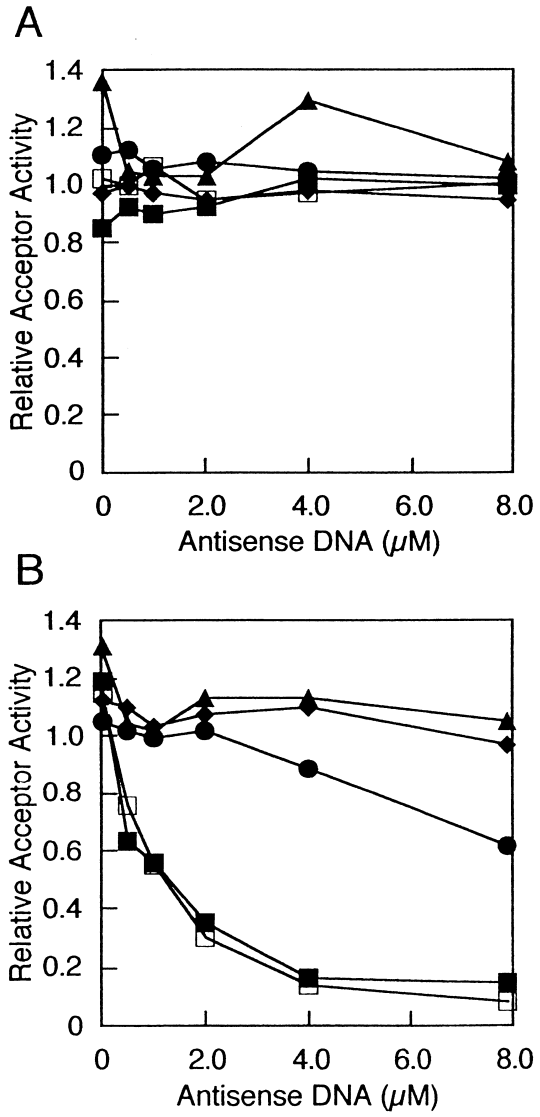

Figure 3. Effects of the treatment of the *Escherichia coli* tRNA mixture in the Mg^{2+}-deficient solution with the $tRNA^{Phe}$-targeted antisense oligonucleotides on the acceptance of Asp (A) and Phe (B). The experimental procedure and the configuration of the figure are the same as in Figure 1, except that $MgCl_2$ was omitted during antisense DNA treatments.

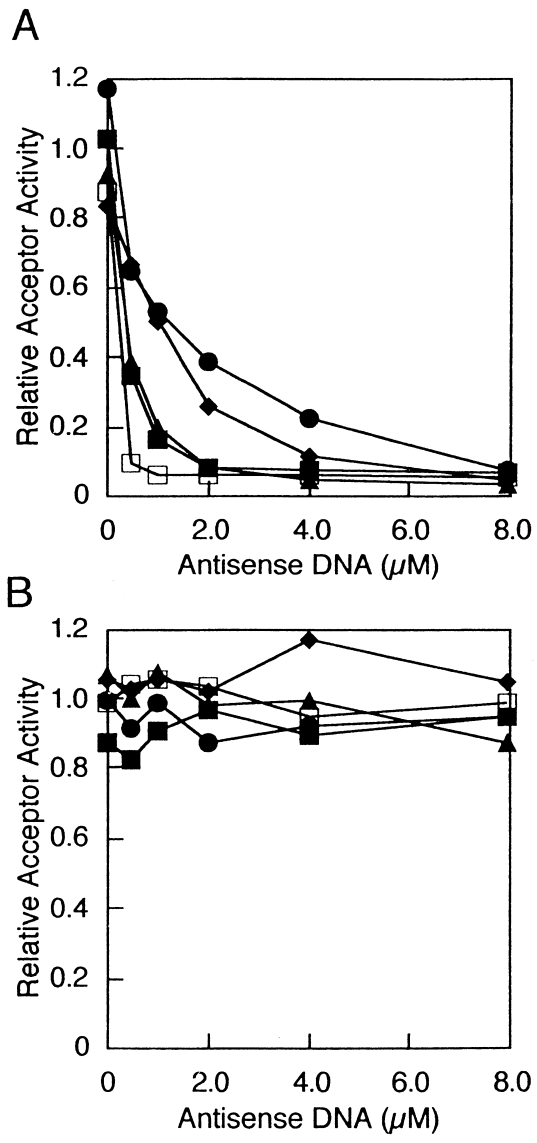

Figure 4. Effects of the treatment of the *Escherichia coli* tRNA mixture in the Mg^{2+}-deficient solution with the $tRNA^{Asp}$-targeted antisense oligonucleotides on the acceptance of Asp (A) and Phe (B). The experimental procedure is the same as in Figure 3, except that different antisense oligomers were used. ■ Asp-1; ● Asp-2; ▲ Asp-3; ◆ Asp-4; □, Asp-5.

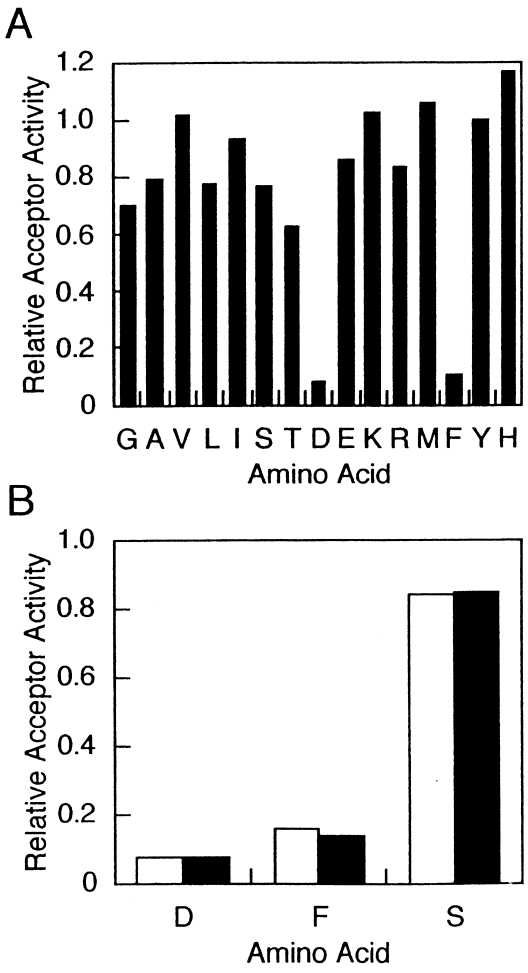

Figure 5. Amino acid-acceptor activities of the *Escherichia coli* tRNA mixture after treatment with Asp-5 and Phe-5. (A) The mixture (169 μL), containing 5.0 nmol Asp-5, 10.1 nmol Phe-5, 0.9 mg *E. coli* tRNA, 2 mM Tris–HCl, 5 mM NaCl, and 0.3 unit/μL RNase H, was incubated and cooled as in Figure 3. The DNAs in this mixture were digested with DNase I (50 units) at 37 °C for 4 h. The tRNA was purified by phenol extraction and ethanol precipitation. The relative acceptor activities of 14 different amino acids were measured as in the preceding figures. (B) The tRNA mixture was further annealed in water (open bars) or in 2 mM Tris–HCl, pH 7.4-5 mM NaCl-1 mM $MgCl_2$ (filled bars), before the measurement of the amino acid-acceptor activities.

Simultaneous inactivation of $tRNA^{Phe}$ and $tRNA^{Asp}$

We further tested if $tRNA^{Phe}$ and $tRNA^{Asp}$ can be inactivated specifically in a single step treatment with Phe-5 and Asp-5 under the Mg^{2+}-deficient conditions (Fig. 5A). The acceptor activities of Phe, Asp, and 12 other amino acids were measured. In this experiment, the antisense DNAs were removed prior to the aminoacylation assay by digestion with DNase. The Phe- and Asp-acceptor activities were lost, as expected. The other 12 amino acids were charged to the tRNA, although the Gly- and Ser-acceptor activities were moderately reduced. This may slightly affect the efficiency of translation with the tRNA mixture. In order to investigate whether the inactivations of $tRNA^{Phe}$ and $tRNA^{Asp}$ were irreversible, the sample was further annealed in water or in a solution containing Mg^{2+} (Fig. 5B). We found that the inactivation of each tRNA was mainly due to the irreversible hydrolysis catalyzed by RNase H. *Thermus thermophilus* RNase H binds a Mg^{2+} ion, according to the crystal structure,[21] and the *E. coli* enzyme requires a Mg^{2+} ion for catalysis.[22] Therefore, it is puzzling that the RNase H was active without Mg^{2+}. However, it is possible that the tRNA-bound Mg^{2+} ions were released during the denaturation and were utilized for the catalysis. It is also possible that Mn^{2+} ions, which may be present in the crude tRNA or the RNase H enzyme solution, activated the RNase H, because the optimum Mn^{2+} concentration for the Mn^{2+}-dependent activity of RNase H is within the micromolar range.[23]

As shown in the previous study, the 'knock out' tRNA mixtures can be used in a cell-free protein synthesis after

the antisense treatments, if the oligonucleotides are removed with DNase.[9] The present study shows that the tRNA composition can be controlled flexibly with the use of antisense oligonucleotides. The Mg^{2+}-deficient conditions may be more useful than the previous conditions. As the target tRNAs are digested by RNase H, it is possible to treat the tRNA further with another antisense DNA under different conditions. For the purpose of constructing a protein synthesis system capable of inserting unnatural/labeled amino acids in multiple positions, the isoacceptor tRNA species that read a limited set of synonymous codons still need to be eliminated. Thus, we are now searching for an antisense DNA that can specifically knock out the AGU/C-specific serine tRNA of *E. coli*. The productivity of cell-free protein synthesis systems has been dramatically improved in recent years.[6,7] It may also be possible to improve the tRNA-dependent cell-free protein synthesis systems. Recently, we have developed an easy method to prepare an S30 extract lacking most of the tRNA.[24] The combination of these systems with the 'knock out' tRNA mixture may provide a new method for preparing engineered proteins.

Experimental

Materials

The antisense oligonucleotides were synthesized by a standard procedure with the use of an Applied Biosystems Model 392 DNA/RNA Synthesizer, and were purified by denaturing 20% polyacrylamide gel electrophoresis followed by electroelution with a Biotrap apparatus (Schleicher & Schuell) and ethanol precipitation.

RNase H from *T. thermophilus* HB8 (*Tth*) was purchased from Toyobo, Japan. The DNase I used for the digestion of the antisense DNAs was RQ1 RNase-free DNase I from Promega.

Aminoacylation assay

The amino acid-acceptor activities of the mixture were measured as described.[9] This was a modification of a standard method.[25]

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