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Polypeptide Synthesis Directed by DNA as a Messenger in Cell-Free Polypeptide Synthesis by Extreme Thermophiles, *Thermus thermophilus* HB27 and *Sulfolobus tokodaii* Strain 7

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Polypeptide synthesis at high temperature directed by single strand DNA as a messenger was investigated using cell-free extracts of an extremely thermophilic bacterium, *Thermus thermophilus* strain HB27, and a hyperthermophilic, acidophilic archaeon, *Sulfolobus tokodaii* strain 7. Aminoglycoside antibiotics enhanced the reaction; neomycin stimulated it most effectively when the extract of the thermophilic bacterium was used, and paromomycin was the best among the antibiotics tested for the extract of the hyperthermophilic archaeon. A common correlation was found between the stimulation of DNA-directed polypeptide synthesis and the misreading rate in RNA-directed polypeptide synthesis. Spermine stimulated the reaction directed by DNA like in the case of poly(Phe) synthesis directed by poly (rU). The cell-free systems can be used for direct production of proteins from genes in high throughput studies on the structural genomics of thermophilus.

Key words: Archaea, cell-free polypeptide synthesis, messenger DNA, *Sulfolobus tokodaii*, *Thermus thermophilus*.

Holland and McCarthy showed that DNA is translated as a messenger in the presence of an aminoglycoside antibiotic in a cell-free system of *Escherichia coli* (*1*). Denatured DNA (*1*) or synthetic polydeoxyribonucleotide (*2*) was used as the template in their experiments. Although the most effective aminoglycoside antibiotic was neomycin, streptomycin and kanamycin have also been reported to be effective (*3, 4*). An antibiotic which caused misreading in mRNA-directed protein synthesis supported the translation of DNA (*4*), but the details of the action mechanism of the antibiotic have not been clarified yet. All these studies were performed with *E. coli* cell-free extracts, and DNA template activity in cell-free polypeptide synthesis by other organisms has not been investigated yet.

To test the generality of the template activity of DNA, polypeptide synthesis directed by DNA in the presence of an antibiotic was investigated using cell-free extracts of *Thermus thermophilus* and *Sulfolobus tokodaii*. *T. thermophilus* is an extremely thermophilic bacterium (eubacterium), and *S. tokodaii* (*5*) is an acidophilic, hyperthermophilic archaeon (archaebacterium), of which the sensitivity to antibiotics for protein synthesis is different from those of eubacteria or eucaryotes (*6*). A general correlation was found in this study between the translation efficiency of DNA and the misreading frequency of mRNA in the presence of an aminoglycoside antibiotic.

Since direct translation of messenger DNA was known to depend on the DNA composition in *E. coli* system (*1, 2*), the effects of the DNA composition were preliminarily studied in the *T. thermophilus* cell-free translation system. Moreover, polyamines are known to be necessary for translation of messenger RNA in the *in vitro* translation system of an extreme thermophiles (*7–10*). We also investigated the effects of polyamines on the translation of DNA as a messenger in the *T. thermophilus* cell-free translation system.

The direct translation of DNA reported here could be used for high throughput studies on thermophile genomes. *In vitro* continuous or semicontinuous protein synthesis has advantages over conventional expression methods (*11, 12*) for the production of proteins to be studied in the field of structural genomics. It has been reported that some thermophile proteins require a high temperature for proper folding (*13*). Thus, it would be desirable to produce thermophile proteins at high temperature. We have already demonstrated that the thermophile cell-free system is stable for up to 6 h at high temperature (*14*) in the presence of appropriate polyamines.

MATERIALS AND METHODS

Reagents and Cultivation—Spermine, poly(rU), poly(dT), neomycin, streptomycin, and paromomycin were purchased from Sigma (Missouri), and ^{3}H-phenylalanine and ^{3}H-leucine were purchased from New England Nuclear (Massachusetts). Transfer RNA mixtures of the thermophiles were prepared according to Zubay (*15*). DNA was prepared from cells of *T. thermophilus* or *E. coli* by phenol extraction and then denatured with 0.2 N NaOH before use. M13mp19 single strand DNA was prepared as described elsewhere (*16*). *T. thermophilus* HB27 and *S. tokodaii* strain 7 were

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grown as described in previous papers (*5, 10*).

Preparation of Cell Free Extracts and Polypeptide Synthesis—S-30 fractions of *T. thermophilus* and *S. tokodaii* were prepared as described previously (*10*).

The standard reaction mixture and the assay procedures for *T. thermophilus* cell-free polypeptide synthesis were the same as previously described (*10*). The reaction mixture for *S. tokodaii* was prepared as described by Friedman (*7*). The final concentration of each of 20 amino acids mixture was 10 μM unless otherwise stated, and the incorporation of ^{3}H-labeled amino acid was determined with a liquid scintillation counter LSC-700 (Aloka, Tokyo).

RESULTS

DNA Directed Amino Acid Incorporation in a T. thermophilus Cell-Free Extract—Amino acids were efficiently incorporated into the hot trichloroacetic acid insoluble fraction in *in vitro* polypeptide synthesis reactions which were directed by denatured DNA and catalyzed by a *T. thermophilus* cell-free extract in the presence of neomycin (Fig. 1a). Streptomycin or gentamycin did not support the reaction efficiently.

DNA Directed Amino Acid Incorporation in a S. tokodaii Cell-Free Extract—The incorporation of amino acids was also investigated using a *S. tokodaii* cell-free extract, in the presence of alkali-denatured DNA as a messenger (Fig. 1b).

The denatured DNA was translated in the presence of paromomycin, but not in the presence of neomycin, streptomycin or gentamycin.

Amino Acid Incorporation Directed by DNA in the Presence of Actinomycin D—Amino acid incorporation directed by DNA of *T. thermophilus* was investigated in the presence of actinomycin D, which is a potent inhibitor of RNA polymerase from the thermophile. In this experiment, 0.5 mM UTP and 0.5 mM CTP were added to the reaction mixture in addition to ATP and GTP.

Amino acid incorporation directed by the double strand DNA extracted from *T. thermophilus* was inhibited by actinomycin D, however, the incorporation directed by the denatured DNA was hardly inhibited by actinomycin D, as shown in Fig. 2a. The incorporation of amino acids without actinomycin D amounted to 0.035 (nmol/mg) (double strand DNA, neomycin = 0 μM) and 0.26 (nmol/mg) (denatured DNA, neomycin = 10 μM), respectively.

Amino acid incorporation directed by the double or single strand DNA of *S. tokodaii* was also investigated in the presence of actinomycin D. Under similar experimental conditions to as described above, similar results were obtained, as shown in Fig. 2b. The incorporation of amino

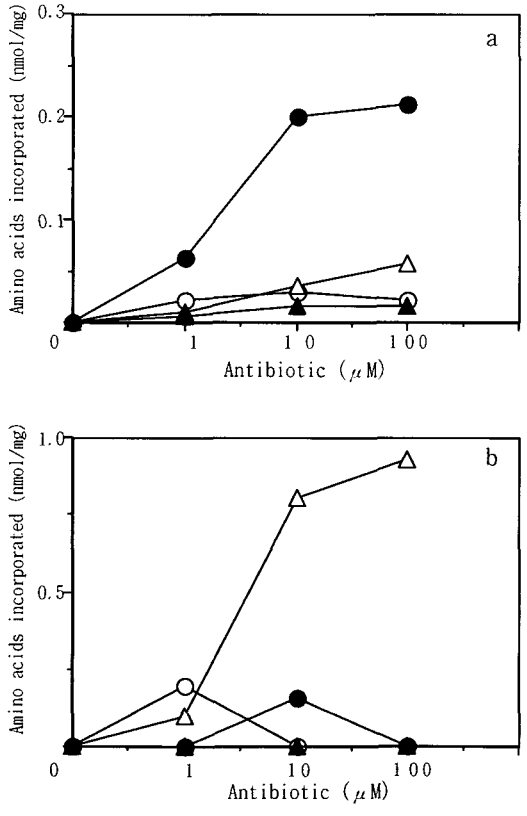

Fig. 1. **Effects of antibiotics on DNA directed polypeptide synthesis catalyzed by cell-free extracts of (a) *T. thermophilus* and (b) *S. tokodaii*.** The reactions were carried out at 65°C for 15 min in the presence of 2 mM spermine. The concentration of each of 20 amino acids was 10 μM. Symbols: ○, streptomycin; ●, neomycin; △, paromomycin; ▲, gentamycin.

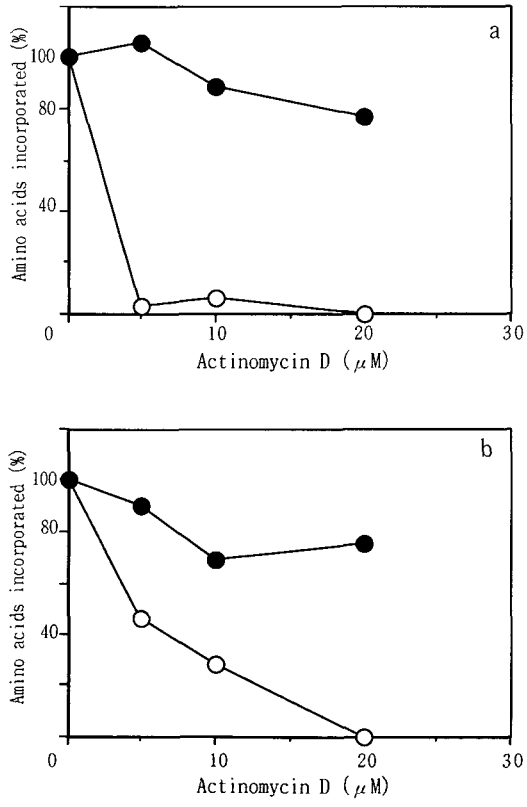

Fig. 2. **Effects of actinomycin D on DNA directed polypeptide synthesis catalyzed by cell-free extracts of (a) *T. thermophilus* and (b) *S. tokodaii*.** The reactions were carried out at 65°C for 15 min in the presence of 2 mM spermine. The results are shown relative to polypeptide synthesis without actinomycin D. The concentration of each of 20 amino acids was 10 μM. Symbols: ○, transcription-translation coupled system directed by double strand DNA; ●, translation system directed by denatured DNA with (a) neomycin or (b) paromomycin.

acids without actinomycin D amounted to 0.70 (nmol/mg) (double strand DNA, paromomycin = 0 μM) and 1.21 (nmol/mg) (denatured DNA, paromomycin = 100 μM) respectively. Although the sensitivity toward actinomycin D was lower in the case of *S. tokodaii* cell-free protein synthesis than that of *T. thermophilus*, the results suggest that the RNA polymerase from the acidophilic, hyperthermophilic archaeon can be suppressed by the antibiotic, which coincides with the previous report (*17*).

Effects of CTP and UTP on Amino Acid Incorporation—To confirm that denatured DNA is directly translated to a polypeptide without transcription to the messenger RNA, the effects of the addition of CTP and UTP on amino acid incorporation directed by the denatured DNA were measured in the presence of neomycin (*T. thermophilus* cell-free extract) or paromomycin (*S. tokodaii* cell-free extract). If the denatured DNA has messenger activity, amino acids will be incorporated into the polypeptide in the presence of actinomycin D without CTP + UTP.

As shown in Table I, amino acids were incorporated in both cell-free extracts in the presence of actinomycin D without CTP + UTP. About 80% amino acid relative to the incorporation without actinomycin D with CTP + UTP was incorporated in the presence of actinomycin D without CTP + UTP in the *T. thermophilus* cell-free extract, and 48% was incorporated in the *S. tokodaii* cell-free extract. These data supported the messenger activity of denatured DNA in the presence of aminoglycoside antibiotics.

Effects of Antibiotics on Misreading—^{3}H-Leucine incorporation was determined in the presence of poly(rU) as a messenger in the *T. thermophilus* cell-free protein synthesis system. The mixture of leucine and phenylalanine was added to the reaction mixture in this experiment.

Leucine incorporation increased with the addition of an antibiotic such as streptomycin, neomycin, paromomycin or gentamycin, as shown in Table II(a). In particular, a large amount of leucine was incorporated with the addition of neomycin.

Similarly, misreading increased with the addition of paromomycin to the *S. tokodaii* cell-free translation system,

TABLE I. **Effect of CTP + UTP on amino acid incorporation in the presence of actinomycin D.** The reactions were carried out at 65°C for 15 min. 10 μM neomycin (for *T. thermophilus* cell-free extract) or 100 μM paromomycin (for *S. tokodaii* cell-free extract) was added to the reaction mixture.

	Amino acid incorporation (nmol/mg-protein)
(a) *T. thermophilus*	
none	0.189 (95%)
+ actinomycin D (20 μM)	0.159 (80%)
+ CTP (0.5 mM) + UTP (0.5 mM)	0.200 (100%)
+ actinomycin D (20 μM)	0.159 (80%)
+ CTP (0.5 mM) + UTP (0.5 mM)	
(b) *S. tokodaii*	
none	0.899 (60%)
+ actinomycin D (20 μM)	0.728 (48%)
+ CTP (0.5 mM) + UTP (0.5 mM)	1.510 (100%)
+ actinomycin D (20 μM)	1.250 (83%)
+ CTP (0.5 mM) + UTP (0.5 mM)	

TABLE II. **Effects of antibiotics on misreading of poly(rU) directed polypeptide synthesis catalyzed by S-30 cell-free extracts of *T. thermophilus* and *S. tokodaii*.** The reactions were carried out at 65°C for 15 min. The rate of misreading is expressed as 100 × (leucine incorporation)/[(phenylalanine incorporation) + (leucine incorporation)].

Antibiotic concentration	Leucine misincorporation (%) 100 × [Leu]/([Phe] + [Leu])
(a) *T. thermophilus*	
none	3.31
streptomycin (5 μM)	4.03
neomycin (5 μM)	8.94
paromomycin (5 μM)	4.02
gentamycin (5 μM)	3.22
(b) *S. tokodaii*	
none	3.53
streptomycin (10 μM)	3.43
neomycin (10 μM)	3.51
paromomycin (10 μM)	5.07
gentamycin (10 μM)	3.15

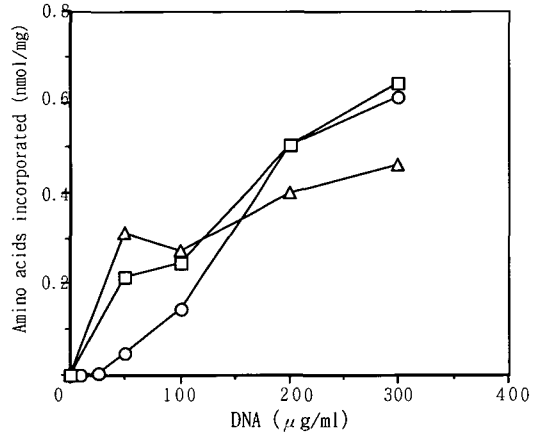

Fig. 3. **Effects of the base compositions of messenger DNAs on polypeptide synthesis catalyzed by S-30 cell-free extracts of *T. thermophilus*.** The reactions were carried out at 65°C for 15 min in the presence of 2 mM spermine and 10 μM neomycin. The concentration of each of 20 amino acids was 10 μM. Symbols: ○, *T. thermophilus* DNA; △, *E. coli* DNA; □, M13mp19 single strand DNA.

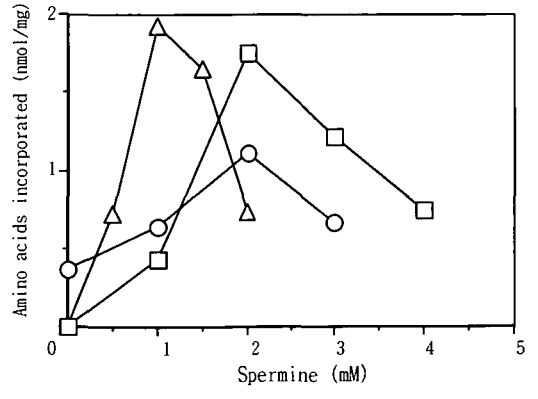

Fig. 4. **Effect of spermine on polypeptide synthesis directed by RNA or DNA catalyzed by S-30 cell-free extracts of *T. thermophilus*.** The reactions were carried out at 65°C for 15 min, and 10 μM neomycin was added to the reaction mixture when DNA was used as a messenger. The concentration of each of 20 amino acids was 10 μM. Symbols: ○, M13mp19 single strand DNA; △, MS2 RNA; □, poly(rU).

as shown in Table II(b). In contrast, streptomycin, neomycin or gentamycin did not affect the translational fidelity. The increase in leucine incorporation in the presence of paromomycin in the *S. tokodaii* cell-free system was smaller than that in the *T. thermophilus* cell-free system in the presence of neomycin.

Effects of the DNA Composition on Translation—It is known that the translation efficiency of DNA varies with its composition in *E. coli* cell-free translation (*2, 18*). To examine the effect of the base composition on *T. thermophilus in vitro* protein synthesis, amino acid incorporation directed by alkaline-denatured chromosomal DNA of *T. thermophilus* or *E. coli*, or single strand DNA of filamentous phage M13mp19 was compared. The G + C content of the DNA of the thermophile is about 70%, and those of the mesophile and phage are about 50%. When each DNA was applied to *in vitro* polypeptide synthesis, it exhibited nearly the same translation efficiency (Fig. 3). The amino acid mixture was incorporated in the hot trichloroacetic acid insoluble fraction proportionally up to 300 μg/ml of DNA.

Effect of Spermine on the Translation of DNA—Unlike the cell-free polypeptide synthesis system of *E. coli*, the systems made with the thermophiles, *T. thermophilus* or *S. tokodaii*, require the addition of a polyamine (*7, 9, 10*). The effect of spermine, which is a commercially available tetramine, was investigated in the presence of M13mp19 DNA, MS2 phage RNA, or poly(rU) as a messenger (Fig. 4).

When M13mp19 single strand DNA was used as the messenger in the presence of neomycin, the optimum concentration of spermine was 2 mM, while when RNAs were used as messengers without neomycin, the optimum concentration of spermine was 1 mM in the case of MS2 phage RNA and 2 mM in the case of poly(rU).

DISCUSSION

DNA directed polypeptide synthesis was observed using a *T. thermophilus* cell-free extract in the presence of an antibiotic. The most effective antibiotic was neomycin, and the optimum concentration of the antibiotic was about 10 μg/ml (≒11 μM). The optimum concentration of neomycin was reported to be 5 μg/ml in a cell-free system of *E. coli* (*18*), and the translation was inhibited with 100 μg/ml of this drug.

The polypeptide synthesis directed by denatured DNA was performed without CTP + UTP in the presence of actinomycin D, which is a potent inhibitor of RNA synthesis by *T. thermophilus*, hence it can be concluded that the polypeptide synthesis in the presence of single-stranded DNA was not mediated by messenger RNA transcribed from the DNA, and the denatured DNA was directly translated in the cell-free extract of *T. thermophilus* in the presence of neomycin, as reported for protein synthesis directed by single strand DNA in the presence of neomycin in an *E. coli* cell-free extract (*19*).

DNA directed polypeptide synthesis was also observed with a *S. tokodaii* cell-free extract in the presence of paromomycin, another aminoglycoside antibiotic. The optimum concentration of paromomycin was 100 μM. Since this protein synthesis by a *Sulfolobus* extract was also performed without CTP + UTP in the presence of actinomycin D, and RNA transcription by DNA-dependent RNA polymerase from *S. acidocaldarius* was reported to be inhibited by this antibiotic (*17*), it can be concluded that the denatured DNA was also translated directly as the messenger.

It has been reported that the misreading in the presence of mRNAs caused by the addition of an antibiotic is correlated with the efficiency of DNA directed polypeptide synthesis mediated by the same antibiotic in an *E. coli* cell-free system (*4*). In the cell-free system of *S. solfataricus*, paromomycin is known to promote misreading of poly(rU) (*20*). In the present experiments, positive correlations were also found between the misreading of poly(rU) and the DNA translation efficiency in cell-free polypeptide synthesis systems of *T. thermophilus* and *S. tokodaii* in the presence of antibiotics. Hence the correlation seems to be a general phenomenon, and not unique to the *E. coli* protein synthesis machinery.

Neomycin was the most effective antibiotic for the translation of DNA using extracts of *E. coli* and *T. thermophilus*, which are bacteria. On the other hand, paromomycin was the most effective for the system of *S. tokodaii*, an archaeon, and it has been reported that in eucaryotes the misreading of codons was enhanced by the addition of paromomycin *in vitro* and *in vivo* (*21, 22*), and in archaea the antibiotic which induces misreading of codons differs from species to species (*20*).

Therefore it may be possible to translate DNA directly as a messenger in an organism by using an antibiotic which induces misreading of poly(rU) most effectively in the organism. Potapov *et al.* reported that a cell-free translation system from *Saccharomyces cerevisiae* could translate poly (dT), but that from wheat germ or rabbit liver could not translate the polydeoxyribonucleotide in the presence of neomycin (*23*). So it may be possible to translate DNA as a messenger directly with cell-free systems from wheat germ and rabbit liver using an antibiotic that induces misreading of poly(rU) effectively in these systems.

In the previous studies, some synthetic DNAs were not efficiently translated directly even in the presence of neomycin in an *E. coli* cell-free translation system (*2, 18*). However, all DNAs used in this work were translated in the presence of this antibiotic when thermophile extracts were used. It has been reported in the case of DNA having a high purine base content that a polypeptide was not synthesized in a cell-free system of *E. coli* directed by the DNA as a messenger (*2, 18*). Perhaps the purine/pyrimidine ratios of the DNAs used in this study were well balanced and they directed polypeptide synthesis efficiently in the presence of neomycin.

In the present study the optimum concentrations of spermine for the translation directed by DNAs were in the same range as observed for the cell-free protein synthesis directed by mRNA. The results suggest that the action of the polyamine is independent of the chemical structures of the sugar moiety of the messenger, either ribose or deoxyribose. The results may imply that activation of translation by spermine depends mainly on ribosomes, ribosomal RNAs or tRNAs. The interaction of the polyamine with messenger DNA or messenger RNA may not be important for activation of translation.

It has been reported that the binding of yeast Tyr-tRNA and Phe-tRNA to the A site of poly(rU_{11}, rA)-programmed *E. coli* ribosomes was stimulated by preincubation of the aminoacyl-tRNAs with spermine, and that misreading of the UUU codon as isoleucine induced by streptomycin was

inhibited by the addition of a polyamine (*24*). On the other hand, Igarashi *et al.* found that misreading caused by paromomycin and kanamycin C in a wheat germ cell-free system did not significantly decreased with the addition of spermidine (*25*). These papers reported the decrease in the misreading with the addition of polyamine. If the misreading is correlated directly with the translation of DNA as a messenger, the decrease in misreading should cause the decrease in the translation of a messenger DNA, thus the addition of a polyamine should lower the activity of DNA as a messenger. In our experiment, however, an increase in the spermine concentration led an increase in the incorporation of amino acids in the translation of DNA as the messenger, hence the correlation between misreading and translation of DNA as the messenger may not be simple. Recently, paromomycin was found to directly bind to the A-site of *E. coli* 16S rRNA and to interfere with translational decoding, inducing misreading (*26, 27*).

From the previous reports and the experiment described in this paper, it is suggested that the polyamine binding site and the site of recognition of the sugar phosphate backbone are closely related but distinct from each other, so that the decrease in misreading caused by a polyamine is not correlated to the decrease in the translation of messenger DNA in the presence of a antibiotic. Further studies are required to clarify the relationship between misreading and translation of DNA as a messenger.

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