T7 Deoxyribonucleic Acid Directed, Rapid-Turnover, Single-Step Addition Reactions Catalyzed by *Escherichia coli* Ribonucleic Acid Polymerase†

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ABSTRACT: The ability of T7 DNA to direct rapid-turnover, single-step addition reactions catalyzed by *Escherichia coli* RNA polymerase has been investigated. In these reactions a nucleoside triphosphate is added to a dinucleoside monophosphate to form a trinucleoside bisphosphate. Only 6 of the 64 possible combinations of substrates gave rapid-turnover reactions according to our criterion for defining these reactions. The six reactions are C-A + UTP → C-A-U, G-A + UTP → G-A-U, C-A + CTP → C-A-C, U-G + UTP → U-G-U, G-U + UTP → G-U-U, and U-A + CTP → U-A-C. Using restriction fragments, we have assigned four of these reactions to known *E. coli* RNA polymerase promoters on T7 DNA. C-A-U is synthesized at the A1 promoter, U-A-C at the C promoter, and both U-G-U and G-U-U at the D promoter. The remaining two reactions were shown to occur outside the early region, possibly at the E or other minor promoters. C-A + UTP → C-A-U is also directed by the A3 promoter, but the reaction is a slow-turnover addition with a rate only one-tenth that of A1-directed C-A-U synthesis. Some of the other 58 combinations of substrates also gave very slow-turnover reactions. One of these slow additions, C-G + CTP → C-G-C, has been assigned to the A2 promoter. Our results indicate that on natural DNA rapid-turnover, single-step addition reactions occur at specific sites, most and possibly all of which are promoters. Not all promoters, however, direct *rapid* single-step additions. The apparent specificity of rapid-turnover, single-step addition reactions may be explained by the relative binding affinities of RNA polymerase to promoter vs. nonpromoter sites along with the low effective concentration of a given trinucleotide coding sequence on natural DNA. Our observation that the same product (C-A-U) is synthesized at significantly different rates on A1 and A3 promoters indicates that rate variations among single-step addition reactions cannot be solely attributed to differences in product formation or dissociation rates. One or more features of the DNA promoter structure outside of the three nucleotides coding for the ribotrinucleotide product must also affect the efficiency of the reaction. Single-step addition reactions should therefore be useful probes in dissecting the influence of DNA sequence on RNA polymerase binding and promoter behavior.

It has been reported that the transcription of certain phage DNAs (T7, T4, T5) by *Escherichia coli* RNA polymerase can be initiated with dinucleoside monophosphate primers instead of a purine nucleoside triphosphate (Downey et al., 1971; Hoffman & Niyogi, 1973; Minkley & Pribnow, 1973; Darlix & Dausse, 1975; Pribnow, 1975a,b). Furthermore, when T7 DNA and RNA polymerase are incubated with certain combinations of a dinucleotide and a nucleoside triphosphate, rifampicin-resistant stable ternary complexes are formed at certain promoters (Minkley & Pribnow, 1973; Dausse et al., 1975). Addition of the three remaining nucleotides to such complexes results in RNA synthesis, with the RNA chain being added on to the trinucleoside bisphosphate formed from the dinucleotide and the nucleoside triphosphate. These results imply that incubation of a dinucleoside monophosphate and a nucleoside triphosphate with RNA polymerase and DNA can result in the formation of a trinucleoside bisphosphate in amounts which are *stoichiometric* with the binary enzyme–DNA complexes. However, because formation of trinucleotides which dissociate very rapidly from the enzyme–DNA complexes probably would not result in any rifampicin-resistant RNA synthesis (Oen & Wu, 1978; McClure & Cech, 1978) and because trinucleotides would not be detected in long-chain RNA analysis, these studies did not investigate the *catalytic* (as opposed to stoichiometric) synthesis of trinucleotide.

In the presence of synthetic DNA templates such as poly[d(A-T)] or poly[d(I-C)], *E. coli* RNA polymerase catalyzes single-step addition reactions in which a nucleoside triphosphate is added to a dinucleoside monophosphate or a trinucleoside bisphosphate (Oen & Wu, 1978). These reactions proceed with a turnover of enzyme until one of the substrates has been used up. In some cases the rate of enzyme turnover is quite rapid. Recently, one such reaction (C-A + UTP → C-A-U) has been reported to occur on a natural DNA template, a λ DNA restriction fragment containing the P_R promoter (McClure & Cech, 1978).

Here we examine the ability of T7 DNA to direct dinucleotide-primed, rapid-turnover, single-step addition reactions. This DNA has three "major" *E. coli* RNA polymerase promoters (A1, A2, and A3) clustered near the left end of the genome (Minkley & Pribnow, 1973; Dunn & Studier, 1973; Chamberlin & Ring, 1972) and several minor promoters (including B, C, D, and E) whose locations have also been mapped (Minkley & Pribnow, 1973; Minkley, 1974; Stahl & Chamberlin, 1977; Kassavetis et al., 1978; McConnell, 1979). The nucleotide sequences of the three major and the

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minor C promoter regions have been determined (Pribnow, 1975a,b; Hsieh & Wang, 1976; McConnell, 1979; Siebenlist, 1979), making this DNA particularly useful for our purposes.

The studies reported here show that catalytic single-step addition reactions are directed by specific sites on this natural DNA and, in some cases, are very rapid. The majority of these rapid-turnover reactions can be assigned to known promoters, and the nonpromoter T7 DNA sequences tested did not direct any of the observed rapid-turnover reactions. Not all promoters, however, direct a *rapid* single-step addition: some display a very slow rate of trinucleotide synthesis. These catalytic single-step addition reactions can provide an assay for the influence of DNA sequences on promoter behavior and catalytic efficiency. They are also useful in the interpretation of results from RNA-synthesizing systems where dinucleotide priming is employed.

Materials and Methods

Chemicals. Ultrapure nucleoside triphosphates were purchased from ICN Pharmaceuticals, Inc. Dinucleoside monophosphates were from Sigma. ^{3}H-Labeled nucleotides were from New England Nuclear or Schwarz/Mann. All other chemicals were reagent grade.

DNA. T7 DNA was prepared as described by Hillel & Wu (1978). T7 ΔD111 DNA was isolated in the same way, except that the phage was grown on *E. coli* C. The ΔD111 deletion phage strain was kindly provided by F. W. Studier.

RNA Polymerase and Other Enzymes. E. coli RNA polymerase was prepared from *E. coli* K12 cells (Grain Processing Corp.) as described previously (Oen & Wu, 1978). The enzyme was >94% pure as shown by $NaDodSO_4$[1] gel electrophoresis (Shapiro et al., 1967) and had a σ content of ~60%. Enzyme activity was assayed as described previously (Wu & Wu, 1978). The specific activity of the enzyme varied from 600 to 900 units/mg. (One unit is defined as the number of nanomoles of tritiated nucleoside monophosphate incorporated in 20 min at 37 °C with calf thymus DNA as template.)

Ribonuclease A (R6KA) and ribonuclease T_1 were purchased from Worthington.

Restriction endonuclease *Hha*I was obtained from Bethesda Research Laboratories, and *Hin*dII was the gift of Dr. Cecilia Guerrier-Takada. *Hpa*II was prepared by the method of Sharp et al. (1973), *Hin*fI was isolated according to a protocol provided by A. Efstradiatis and T. Maniatis, and *Dpn*II was prepared according to Lacks & Greenberg (1977).

Initial Screening Experiments for Rapid-Turnover Reactions with T7 DNA. Fifty microliters of solution I containing 0.08 M Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 50 mM KCl, 0.01 M dithiothreitol, 0.1 A_{260} unit (0.2 pmol) of T7 DNA, and 7 μg (14 pmol) of RNA polymerase was incubated for 5 min at 37 °C and then mixed with 50 μL of solution II which contained 5 mM $MgCl_2$, 50 mM KCl, 20 μM dinucleoside monophosphate, and 10 μM ^{3}H-labeled nucleoside triphosphate (500–1500 cpm/pmol). The resultant solution was incubated for another 10 min at 37 °C. The reactions were stopped by chilling the mixture on ice and adding EDTA to a final concentration of 50 mM.

Analysis of Single-Step Addition Reaction Mixtures. Ten-microliter aliquots of reaction mixtures were streaked at the origin of $^1/_2$-in. wide strips of Whatman 3 MM paper and developed, unless otherwise indicated, by ascending chromatography with the WASP solvent described by Johnston & McClure (1976). The chromatograms were then air-dried, cut into 1-cm strips, and counted in Econofluor (New England Nuclear) in a Beckman liquid scintillation counter.

Preparation and Isolation of T7 DNA Restriction Fragments. All restriction fragments were derived from wild-type T7 DNA. The nomenclature for these fragments is illustrated by the following examples: $(HpaII/HpaII)_{104}$ is a 104 base pair fragment of T7 DNA cut on both ends by endonuclease *Hpa*II; $(HhaI/HinfI)_{436}$ is a 436 base pair fragment generated from T7 DNA and having a *Hha*I cut on the left end and a *Hin*fI cut on the right end; $(LHE/HinfI)_{1100}$ contains the left-hand end of T7 DNA (LHE) and a *Hin*fI cut on the right side of the restriction piece. The sizes of some of the fragments used in these studies were previously established by electrophoretic mobility or total sequence analysis (Hsieh & Wang, 1976; Siebenlist, 1979; R. Haas and P. Cole, unpublished experiments). The lengths of other fragments were determined by using *Hin*dII fragments of λ DNA (Maniatis et al., 1975) and T7 DNA *Hpa*II and *Hin*dII fragments as molecular weight standards.

The methods for isolating and purifying the fragments used in this study are the same as those described by P. Cole and R. Haas (unpublished experiments). Homogeneity and intactness of the fragments were confirmed by using native and denaturing analytical polyacrylamide gels (Peacock & Dingman, 1968; Maxam & Gilbert, 1977). The optical purity of each fragment was checked by determining the A_{260}/A_{280} and A_{260}/A_{230} spectral ratios, which agreed with values reported for pure DNA (Thomas & Abelson, 1966). Concentrations of T7 DNA and DNA fragments were determined by absorbance measurements assuming that the absorbancy of DNA at 260 nm is 20 mg^{-1} cm^2.

Results

Initial Screening for Rapid-Turnover, Single-Step Addition Reactions. In order to determine whether any rapid-turnover, single-step addition reactions could be directed by T7 DNA, we used an initial screening procedure in which all possible combinations of a dinucleoside monophosphate and a single ^{3}H-labeled nucleoside triphosphate (there are 64 such combinations) were incubated with the DNA and RNA polymerase. The following conditions for the incubations were chosen in order to maximize the chances of observing such reactions. DNA and enzyme were preincubated for 5 min at 37 °C before addition of the substrates, and a large molar excess of enzyme over DNA was used so that all major and minor promoter sites, and potentially some nonspecific sites as well, would be occupied (Stahl & Chamberlin, 1977). We also used low concentrations of KCl (50 mM) so that the E promoter [which is not utilized for transcription at KCl concentrations of 100 mM or higher (Stahl & Chamberlin, 1977)] would be functional. Finally, the concentration of nucleoside triphosphate employed was low enough (5 μM) to prevent initiation by ATP or GTP.

The reaction mixtures were analyzed by paper chromatography using the WASP solvent system of Johnston & McClure (1976) to see if any significant amounts of trinucleotide were being formed. In this solvent system all four nucleoside triphosphates move well away from the origin. The lowest mobility nucleoside triphosphate species is ATP, which has an R_f of 0.4. Any trinucleoside bisphosphate would be expected to move quite close to the origin ($R_f < 0.2$). For several combinations of substrates, analysis of the reaction mixture did show formation of radioactive material with an R_f less than 0.2. A reaction in which at least 10% of the nucleoside triphosphate is converted into a product which moves close to the chromatogram origin after a 10-min, 37

[1] Abbreviation used: $NaDodSO_4$, sodium dodecyl sulfate.

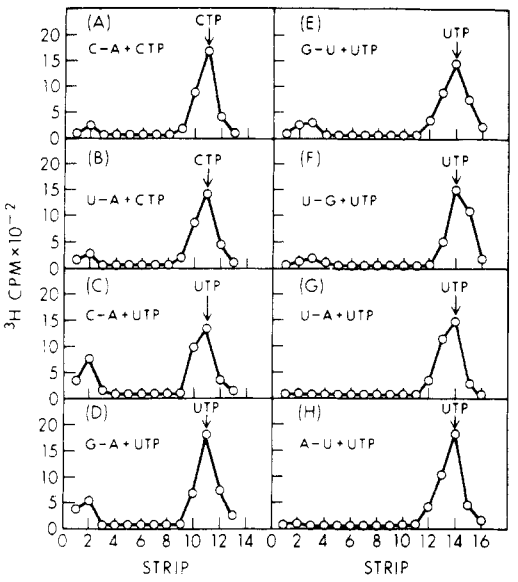

FIGURE 1: Paper chromatographic analysis of single-step addition reactions directed by T7 DNA. Each reaction mixture contained a dinucleotide and an ^{3}H-labeled nucleoside triphosphate as indicated. The mixtures were prepared, incubated, and analyzed as described under Materials and Methods. Strip no. 1 of each chromatogram contains the origin.

°C incubation under our conditions is designated a *rapid-turnover, single-step addition reaction.* (Ten percent conversion of nucleoside triphosphate into product during this time period corresponds to a product formation rate of 50 mol (mol of T7 DNA)$^{-1}$ min^{-1}.) The combinations of substrates which gave such reactions are C-A + UTP, G-A + UTP, G-U + UTP, U-G + UTP, U-A + CTP, and C-A + CTP.

Paper chromatographic analyses of reaction mixtures containing these six combinations of substrates are shown in Figure 1. For comparison, two other combinations of substrates, A-U + UTP and U-A + UTP, which did not give rapid-turnover reactions according to our arbitrary criterion are also shown. In both of these latter reaction mixtures, however, some trinucleotide, either U-A-U or A-U-U, was detectable. If the incubation period was increased to 1 h, more than 10% of the [^{3}H]UTP was converted to the corresponding trinucleotide. Thus, single-step addition reactions did seem to occur with these two combinations of substrates, but the rate of formation of product was much slower than that obtained in the six rapid-turnover reactions. Many of the remaining 56 combinations of substrates also appeared to give these slower single-step addition reactions. These reactions were not studied further, with one exception (C-G + CTP), which will be discussed later.

In all cases the appearance of radioactivity near the origin of the chromatograms was absolutely dependent on the presence of DNA in the reaction mixture. The catalytic single-step addition reactions were essentially linear with time until the nucleoside triphosphate was nearly exhausted. Typically, greater than 90% of the nucleoside triphosphate could be converted to trinucleotide by extended incubations. Omission of the 5-min preincubation of RNA polymerase with the DNA did not appreciably affect the results. As part of the initial characterization of these reactions, the six rapid-turnover reactions were carried out at 50, 100, and 150 mM KCl. The results indicated that over this concentration range there is little, if any, effect of salt on the efficiency of these reactions. Unless otherwise indicated, the single-step addition reactions were routinely carried out at 100 mM KCl in our subsequent studies.

Characterization of the Products. The six rapid-turnover reactions detected in our initial screening experiments presumably resulted in the formation of the trinucleotides G-A-U, C-A-U, U-A-C, C-A-C, G-U-U, and U-G-U. In principle, however, longer products could also be formed if more than one nucleotide residue was added to the dinucleotide initiator or if the substrates contained other nucleotides as impurities. We therefore subjected the products of the six rapid-turnover reactions to further analysis. Reaction mixtures analogous to those of Figure 1 were incubated for 1 h rather than 10 min to prepare sufficient product for detailed characterization. Aliquots of these reaction mixtures were analyzed by descending paper chromatography in WASP solvent overnight to enhance the resolution of products. Each reaction mixture gave one major product peak which had moved away from the origin and which accounted for most of the radioactivity other than starting material. In some cases there were also some very minor peaks closer to or at the origin.

In order to determine whether these smaller peaks corresponded to longer chain RNAs, we examined the effect of rifampicin on the rapid-turnover reactions under the 1-h incubation conditions described above. Rifampicin inhibits elongation of RNA chains but still permits the synthesis of a trinucleoside bisphosphate from a nucleoside triphosphate and a dinucleoside monophosphate (Oen & Wu, 1978; McClure & Cech, 1978). When the reaction mixtures contained 12 μM rifampicin, the major peaks were reduced in size by 40–70% and the smaller peaks were eliminated. The presence of rifampicin in the reaction mixtures had no effect on the R_f of the major peak. The decreased yield was probably due to a decreased affinity of the substrates for RNA polymerase (McClure & Cech, 1978). The minor peaks were presumably RNA chains longer than trinucleotides.

To confirm that the main product peaks were trinucleotides with the expected structures, we took aliquots of the reaction mixtures which had been incubated with rifampicin and digested them with 0.6 μg/mL RNase A for 10 min at 37 °C. The digestion products were then analyzed by paper chromatography in WASP solvent. In addition, aliquots of the reaction mixtures containing the putative trinucleotides U-G-U, G-U-U, and G-A-U were also digested with 0.6 μg/mL RNase T_1 for 10 min at 37 °C and the products similarly analyzed. In each case (except for the treatment of G-A-U with RNase A which produced no change), the radioactive digestion products displayed mobilities different from those of the undigested material. The mobilities of the digested products were the expected ones as indicated by comparison with the appropriate dinucleotide markers (data not shown). For example, the putative U-G-[^{3}H]U was quantitatively converted to a peak with the R_f of G-U when digested by RNase A and one with the R_f of uridine when digested by RNase T_1. Thus, the products of the six rapid-turnover reactions are in fact the expected trinucleotides.

Rapid-turnover, single-step addition reactions occur at specific sites on T7 DNA. We wanted to determine whether the six observed rapid-turnover reactions were being directed by polymerase molecules bound at specific sites on the DNA (i.e., high-affinity binding sites such as promoters) or whether the reactions were being catalyzed by enzyme molecules bound at nonspecific sites on the DNA. Experiments designed to answer these questions were carried out with T7 DNA restriction fragments and with DNA from the T7 mutant ΔD111.

Since the major promoters A2 and A3 as well as the minor promoter B are deleted in ΔD111 DNA (Studier, 1975), we

Table I: Ability of T7 DNA Restriction Fragments to Direct Rapid-Turnover, Single-Step Addition Reactions[a]

fragment	promoters contained on fragment[b]	product					
		C-A-U	G-U-U	U-G-U	G-A-U	U-A-C	C-A-C
(*Hin*dII/*Hin*dII)$_{281}$	A1	+	−	−	−	−	−
(*Hin*dII/*Hin*dII)$_{104}$	A2	−	−	−	−	−	−
(*Hpa*II/*Hpa*II)$_{104}$	A2	−	−	−	−	−	−
(*Hin*dII/*Hin*dII)$_{120}$	none	−	−	−	−	−	−
(LHE/*Hin*fI)$_{1100}$	D, A1, A2, A3	+	+	+	−	−	−
(LHE/*Hpa*II)$_{520}$	D, A1	+	+	+	−	−	−
(*Hha*I/*Hin*fI)$_{436}$	A3	(+)	−	−	−	−	−
(LHE/*Hha*I)$_{638}$	D, A1, A2	+	+	+	−	−	−
(*Hin*fI/*Hin*fI)$_{400-460}$	none	−	−	−	−	−	−
(LHE/*Dpn*II)$_{8268}$	D, A1, A2, A3, B, C	+	+	+	−	+	−

[a] Reaction mixtures contained, in a total volume of 40 μL, 50 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 100 mM KCl, 5 mM dithiothreitol, 10 μM dinucleotide, 5 μM [^{3}H]UTP or CTP (500–1500 cpm/pmol), 0.07 pmol of DNA fragment *or* 0.72 μg of the *Hin*fI digest mixture (see text), and 15 pmol of RNA polymerase. Each mixture was incubated 1 h at 37 °C, and then EDTA was added to a concentration of 30 mM. 10-μL aliquots of each reaction mixture were analyzed by paper chromatography as described under Materials and Methods. The + sign indicates above-background radioactivity detected on the chromatogram at the position corresponding to the appropriate trinucleotide. The − sign indicates that no radioactivity was found at this position. The (+) sign indicates a detectable above-background radioactivity at the trinucleotide position but at a lower level as compared with other samples (see text). [b] Assignment of promoters to the various restriction pieces is based on the data of Minkley & Pribnow (1973), Hsieh & Wang (1976), McDonell et al. (1977), Stahl & Chamberlin (1977), Siebenlist (1979), and the unpublished results of P. Cole and R. Haas.

examined the ability of this DNA to direct the six rapid-turnover reactions observed with wild-type T7 DNA. When T7 and ΔD111 DNA were incubated with the six combinations of substrates and RNA polymerase under identical reaction conditions, both templates gave essentially the same results (Figure 2). Thus, since all the reactions directed by wild-type T7 DNA are also directed by ΔD111 DNA, none of the rapid-turnover, single-step additions can be occurring *exclusively* at the A2, A3, or B promoters. Stahl & Chamberlin (1977) have reported that transcription of ΔD111 DNA occurs primarily from the A1 promoter at a low RNA polymerase/DNA ratio (~1:1). When the substrates for the six rapid-turnover reactions were incubated with enzyme and ΔD111 DNA under such conditions, only one of the reactions was observed, C-A + UTP → C-A-U (Figure 2). It therefore seems reasonable that this reaction occurs at the A1 promoter. This result, however, does not rule out its occurrence at other sites on the DNA as well.

Better evidence that the rapid-turnover reactions occur at promoters and not at nonspecific sites was obtained with restriction fragments. A typical experiment is presented in Figure 3. This figure shows the results of incubations of the substrates C-A + UTP with RNA polymerase and (A) T7 DNA, (B) (*Hin*dII/*Hin*dII)$_{281}$ (containing the A1 promoter), (C) (*Hin*dII/*Hin*dII)$_{104}$ (containing the A2 promoter), or (D) (*Hin*dII/*Hin*dII)$_{120}$ (containing no promoter). Only the samples containing T7 DNA or (*Hin*dII/*Hin*dII)$_{281}$ show a significant formation of C-A-U. In all such experiments, a single-step addition reaction directed by a DNA restriction fragment was less efficient than the same reaction directed by an equal molar amount of T7 DNA. The reason for the difference in efficiency is not clear; we cannot rule out the trivial explanation that the fragments were somewhat damaged in an undetectable way during preparation.

Table I summarizes the results of experiments carried out with T7 DNA restriction fragments. The possibility that any of the rapid-turnover, single-step addition reactions could be occurring at nonspecific sites on the DNA seems to be ruled out by the observation that the DNA fragments do possess a great deal of selectivity in directing these reactions. The fragments (*Hin*dII/*Hin*dII)$_{104}$ and (*Hpa*II/*Hpa*II)$_{104}$ as well as the mixture (*Hin*fI/*Hin*fI)$_{400-460}$ did not give any of the six rapid-turnover, single-step addition reactions. On the other hand, all of the fragments which *did* direct at least one of the

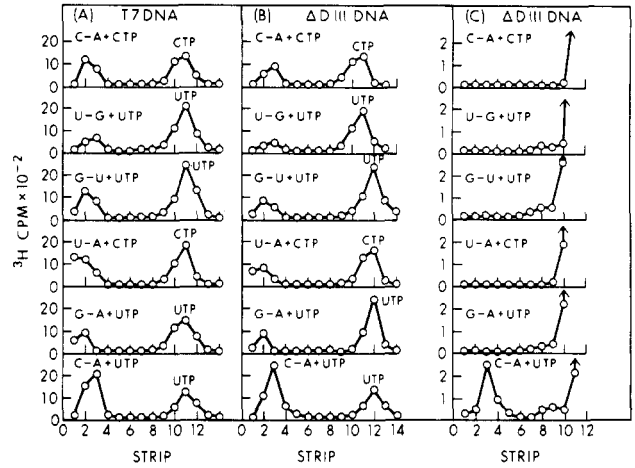

FIGURE 2: Comparison of rapid-turnover, single-step addition reactions directed by T7 and ΔD111 DNAs at a high enzyme/DNA ratio and by ΔD111 DNA at a low enzyme/DNA ratio. Reaction mixtures contained, in a volume of 40 μL, (A) 0.036 A_{260} unit (0.07 pmol) of T7 DNA and 6 μg (12 pmol) of RNA polymerase (enzyme/DNA ratio ~170), (B) 0.036 A_{260} unit (0.07 pmol) of ΔD111 DNA and 6 μg (12 pmol) of RNA polymerase (enzyme/DNA ratio ~170), and (C) 0.072 A_{260} unit (0.14 pmol) of ΔD111 DNA and 0.5 μg (0.1 pmol) of RNA polymerase (enzyme/DNA ratio ~0.7). The mixtures were incubated for 15 min at 37 °C. Otherwise they were prepared and analyzed like those in Figure 1.

rapid-turnover reactions did contain at least one promoter.

As can be seen in Table I, all of the fragments with the A1 promoter direct the reaction yielding C-A-U. The fragment containing only the A3 promoter, (*Hha*I/*Hin*fI)$_{436}$, can also direct this reaction, but the efficiency is much lower. When parallel incubations were carried out with equimolar amounts of the fragments (*Hha*I/*Hin*fI)$_{436}$ containing A3 and (*Hin*dII/*Hin*dII)$_{281}$ containing A1, only 10% as much C-A-U was formed in the reaction mixture containing the former fragment as in the one containing the latter fragment. The extent of reaction obtained with a mixture of the two fragments is essentially the sum of what was obtained with each one separately. This indicates that the lower efficiency of the A3 fragment in directing this reaction could not have been due to an inhibitor contaminating the fragment preparation. Also, the efficiencies of these two fragments in directing these reactions did not change as the KCl concentration was varied from 50 to 200 mM.

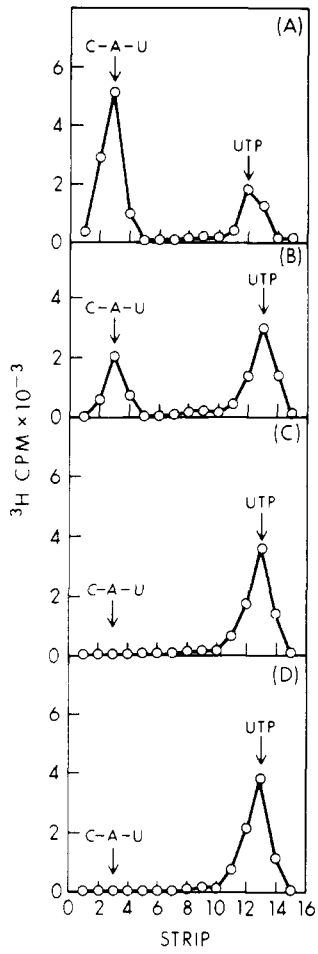

FIGURE 3: Comparison of the ability of T7 DNA and various restriction fragments to direct the reaction C-A + UTP → C-A-U. 40-μL reaction mixtures were prepared with 0.07 pmol of either (A) wild-type T7 DNA, (B) (*Hin*dII/*Hin*dII)$_{281}$ containing the A1 promoter, (C) (*Hin*dII/*Hin*dII)$_{104}$ containing the A2 promoter, or (D) the nonpromoter fragment (*Hin*dII/*Hin*dII)$_{120}$ plus 10 μM C-A and 5 μM [^{3}H]UTP (1000 cpm/pmol). Other components, incubation conditions, and analysis were as described in Table I.

All of the fragments containing the D promoter directed single-step addition reactions yielding the products G-U-U and U-G-U, while the fragments lacking this promoter could not direct either of these reactions. Thus, these two reactions are probably catalyzed by RNA polymerase molecules bound at the D promoter and nowhere else.

Similarly, it is likely that the rapid-turnover reaction U-A + CTP → U-A-C is catalyzed only by RNA polymerase bound at the C promoter because among the ten fragments that we studied only (LHE/*Dpn*II)$_{8268}$ was able to direct this reaction. This fragment was also the only one containing the C promoter.

Some of the T7 promoters (A2 and B) do not direct any of the rapid-turnover, single-step addition reactions (Table I). Moreover, even though the A3 promoter does direct the reaction yielding C-A-U, the rate of appearance of product is about an order of magnitude lower than when the same reaction is directed by the A1 promoter. Thus, the A3 promoter cannot truly be said to direct a rapid-turnover reaction. These results can explain the fact that no difference was seen in either the number or efficiency of the rapid-turnover, single-step addition reactions directed by T7 DNA or by ΔD111 DNA; the promoters which are missing on the ΔD111 DNA (A2, A3, B) are those which do not direct rapid-turnover, single-step addition reactions, so their absence has no effect.

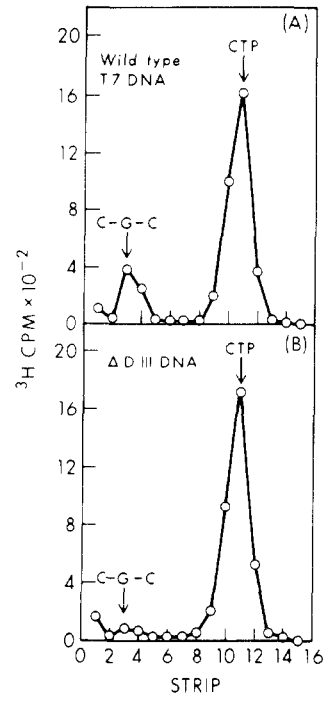

FIGURE 4: Comparison of the ability of T7 DNA and ΔD111 DNA to direct the single-step addition reaction C-G + CTP → C-G-C. 40-μL reaction mixtures contained 0.024 A_{260} unit (0.048 pmol) of T7 DNA (A) or ΔD111 DNA (B) and 7.5 μg (15 pmol) of RNA polymerase. The KCl concentration was 150 mM, and incubation of the complete mixtures was for 1 h at 37 °C. Otherwise they were prepared and analyzed like those in Figure 1.

The data in Table I do not allow the assignment of two of the rapid-turnover reactions (those yielding C-A-C and G-A-U) to any known T7 promoter. They do appear to come from sites outside the early region since neither was directed by the fragment (LHE/*Dpn*II)$_{8268}$ which contains the entire early region (McDonell et al., 1977). Whether the E promoter, which lies outside the early region, directs one or both of these reactions remains unclear. Although this promoter is not transcribed at salt concentrations of 100 mM KCl or higher (Stahl & Chamberlin, 1977), we observe that all six rapid-turnover, single-step addition reactions occur equally well between 50 and 150 mM KCl. However, the salt dependence of a single-step addition reaction may be quite different from that of transcription from a given promoter. Our observations, therefore, are not sufficient to eliminate the E promoter as a site which directs C-A-C or G-A-U synthesis. In any case, it seems likely that these reactions are directed by a specific site(s) outside the early region of T7 DNA.

The Slow-Turnover, Single-Step Addition Reaction C-G + CTP → C-G-C. As mentioned above, the A2 promoter does not direct any of the rapid-turnover, single-step addition reactions according to our criterion for rapid-turnover reactions. We wanted to see if RNA polymerase bound at this promoter could catalyze a slower single-step addition reaction. A likely candidate for such a reaction based on the known sequence of this promoter (Pribnow, 1975b) is C-G + CTP → C-G-C. As shown in Figure 4, this reaction is carried out by RNA polymerase in the presence of T7 DNA. Its rate, however, is much slower than the rate of any of the six rapid-turnover reactions. In the presence of excess enzyme, the rate of formation of C-G-C is ~13 mol (mol of T7 DNA)$^{-1}$ min^{-1} as compared to 100 mol (mol of T7 DNA)$^{-1}$ min^{-1} for C-A-U formation. If ΔD111 DNA, which lacks the A2 promoter, is used in place of wild-type T7 DNA, significantly less C-G-C is formed (Figure 4). Thus, it seems that

Table II: Ability of T7 DNA Restriction Fragments to Direct the Slow-Turnover, Single-Step Addition Reaction C-G + GTP → C-G-C[a]

fragment	promoter contained on fragment[b]	ability to direct reaction
$(HindII/HindII)_{281}$	A1	−
$(HindII/HindII)_{104}$	A2	+
$(HpaII/HpaII)_{104}$	A2	+
$(HindII/HindII)_{120}$	none	−
$(LHE/HinfI)_{1100}$	D, A1, A2, A3	+
$(LHE/HpaII)_{520}$	D, A1	−
$(HhaI/HinfI)_{436}$	A3	−
$(LHE/HhaI)_{638}$	D, A1, A2	+
$(HinfI/HinfI)_{400-460}$	none	−

[a] Reaction mixtures containing 10 μM C-G and 5 μM [^{3}H]CTP (1500 cpm/pmol) were prepared, incubated, and analyzed exactly like those described in footnote *a* of Table I. The + sign indicates a radioactive peak with the R_f of C-G-C detected on the chromatogram. The − sign indicates that no above-background radioactivity was found at this position. [b] See Table I for promoter content assignment.

the A2 promoter is responsible for most of the C-G-C formation directed by T7 DNA. As shown in Table II, experiments with restriction fragments confirmed that this reaction is in fact directed by the A2 promoter.

Discussion

Although it has been reported that the incubation of T7 DNA and RNA polymerase with a dinucleotide plus a single nucleoside triphosphate can lead to the formation of a stable ternary (initiation) complex at specific sites on the DNA (Minkley & Pribnow, 1973; Dausse et al., 1975), we have shown that such an incubation can have another result as well, namely, the *catalytic* (as opposed to stoichiometric) formation of a trinucleotide. Interestingly, these catalytic reactions showed substantial selectivity since only 6 out of 64 possible combinations of substrates (i.e., C-A + UTP, G-A + UTP, U-A + CTP, C-A + CTP, G-U + UTP, and U-G + UTP) gave rise to a rapid synthesis of trinucleotides on T7 DNA.

What contributes to the specificity and high turnover number of these single-step addition reactions on a natural DNA template? In the case of the dinucleotide-primed, single-step addition reactions directed by poly[d(A-T)] (Oen & Wu, 1978), it was found that the products had to be completely complementary to the DNA (i.e., U-A-U could be formed but not A-U-U). We therefore expected that the single-step additions templated by T7 DNA would likewise be directed *only* by sequences on the DNA complementary to the ribotrinucleotide product. While this expectation seems to be correct (see below), we found that two additional factors contribute to the selectivity of the rapid-turnover, single-step addition reactions. First, these reactions are directed by specific sites on the DNA; i.e., nonpromoter fragments do not direct such reactions, and four of the six reactions can be assigned to known promoters (see Table III). Second, some single-step addition reactions (such as those directed by A2 and A3 promoters) occur *too slowly* to meet our criterion for a rapid-turnover reaction.

Table III summarizes the promoter assignments for several single-step addition reactions. Insofar as the sequences are known, this table indicates that each promoter does, as expected, contain the correct complementary sequence to direct the formation of the assigned ribotrinucleotide product. Moreover, the reactions directed by A1, A2, A3, and C promoters all yield trinucleotides whose middle nucleotide is

Table III: Summary of Promoter Assignments for Single-Step Addition Reactions[a] and Comparison of Trinucleotide Product Sequences with DNA Promoter Sequences

reaction	promoter capable of directing reaction	promoter sequence[d]
C-A + UTP → C-A-U[b]	A1	ACAGCCATCGAGA
	A3	TACCACATGAAAC
U-A + CTP → U-A-C	C	TATCTTACAGGTC
U-G + UTP → U-G-U	D	
G-U + UTP → G-U-U	D	
C-G + CTP → C-G-C[c]	A2	CAAATCGCTAGGT
C-A + CTP → C-A-C	unassigned	
G-A + UTP → G-A-U	unassigned	

[a] We cannot, of course, rigorously exclude the possibility that the four assigned reactions in this table may also occur at specific (promoter) sites elsewhere on the DNA since quantitation of total synthesis for a given trinucleotide on fragments as compared with intact DNA is not feasible. [b] The rate of A3-directed C-A-U synthesis is approximately one-tenth that of A1-directed C-A-U synthesis. [c] This reaction is a slow-turnover, single-step addition (see text). [d] The DNA strand sequence given is the one identical in sense to the RNA transcript. The underlined nucleotide corresponds to the normal initiating nucleotide for transcription. The A2 and A3 sequences are from the data of Pribnow (1975a,b); A1 and C are from the data of Siebenlist (1979) and McConnell (1979), respectively.

the normal start site for transcription. Whether the trinucleotide product must always be precisely positioned about the transcription initiation point, however, is not clear. This implied positioning requirement is strengthened by the observation that C-A + CTP → C-A-C is not directed by A3 (see below) even though that sequence is centered only two nucleotides to the left of the A3 initiation point. The available data on the D promoter, however, suggest some positioning flexibility. The D promoter sequence about the pppG initiation site was postulated by Minkley & Pribnow (1973) to be U-G-U, yet we found reactions yielding both U-G-U and G-U-U. Perhaps an extended inferred sequence for the D promoter is U-G-U-U.

In all of the cases where we were able to assign a single-step addition reaction to a promoter of known sequence (i.e., those occurring at the A1, A2, A3, and C promoters), the next nucleotide called for by the DNA sequence is different from the one which is added to the dinucleotide. Therefore, RNA synthesis stops after one bond has been formed simply because of the lack of the next nucleotide. We believe, however, that such a situation is not always a required condition for the generation of a trinucleotide from a rapid-turnover, dinucleotide-primed, single-step addition reaction. For example, it has been observed (H. Oen, unpublished experiments) in a poly[d(A-T)]-directed system that incubation of the substrates U-A, UTP, and 3′-deoxy-ATP (3′-dATP) with RNA polymerase results in the formation of U-A-U-3′-dA. Nonetheless, it was found that the trinucleotide U-A-U could still be the *major* product, depending on reaction conditions. Consistent with this type of situation is our proposal that the sequence of the RNA from the D promoter is U-G-U-U even though the major product of a U-G + UTP incubation was U-G-U. In the case of abortive initiation directed by the $\lambda P_R'$ promoter, McClure et al. (1978) found that, in the presence of ATP, CTP, and GTP, the product pppA-A-C is formed even though the DNA coding potentially allows the sequence A-A-C-G to be synthesized. These authors concluded that at least in vitro very short RNA chains may be as likely to dissociate from the enzyme–DNA complex as to be elongated. It is interesting to note that in all of the *rapid* T7 DNA-

directed, single-step additions reported here a pyrimidine is the added mononucleotide. In the case of a poly[d(A-T)] template (Oen & Wu, 1978), the U-A + UTP → U-A-U reaction is much more rapid than A-U + ATP → A-U-A. Thus, having a 3′-terminal pyrimidine in the trinucleotide product may in general facilitate the release of product from the enzyme–DNA complex. The single-step addition of a pyrimidine mononucleotide to a dinucleotide is not, however, sufficient in itself for *rapid* synthesis of trinucleotide because other reactions involving pyrimidine mononucleotide addition are slow (the A2- and A3-directed reactions, for example). We cannot say from currently available data whether addition of a purine mononucleotide to a dinucleotide is always a slow-turnover reaction.

What causes single-step addition reactions to occur at different rates at different promoters? One possibility is that there are intrinsic differences in the rate at which RNA polymerase can catalyze the formation of trinucleotides of different sequences. If the rate-determining step in the reaction is the dissociation of product, the differences could be due to different affinities of the trinucleotides for the enzyme. Our observation, however, that the formation of the same trinucleotide (C-A-U) occurs much more rapidly at the A1 than at the A3 promoter indicates that other factors must be involved. This finding implies that it is not just the structure (sequence) of the RNA product but rather some feature of the DNA structure in the promoter region outside of the three bases complementary to the ribonucleotide product which determines the efficiency of the reaction.

The observation that nonpromoter fragments do not yield detectable amounts of rapid-turnover, single-step addition products in the presence of excess polymerase could be explained by the relative binding affinities of RNA polymerase to promoter vs. nonpromoter sites (Chamberlin, 1976) and by the effective concentration of a given trinucleotide coding sequence on natural DNA. Dinucleotide-primed, rapid-turnover, single-step addition reactions have previously been observed with "synthetic" nonpromoter DNAs such as poly[d(A-T)] and poly[d(I-C)] (Oen & Wu, 1978). For natural DNA, however, the effective concentration of sequences complementary to a given trinucleotide product is substantially lower. Therefore, even with considerable nonspecific binding, the fraction of polymerase molecules bound to any particular trinucleotide sequence is very small. In contrast, the binding constant for a polymerase–promoter complex could be sufficiently great compared with nonpromoter binding to allow much more synthesis of the trinucleotide product which is complementary to an appropriately positioned coding sequence. Alternatively, it is possible that some specific structural configuration of the promoter which is required for catalysis is missing in the nonpromoter fragments.

The tentative trend indicated by our results with T7 DNA is the following. Rapid-turnover, single-step addition reactions on natural DNA are promoter site directed, but not all promoters direct rapid-turnover, single-step additions. We cannot say from the present study whether there are natural DNA sites other than promoters which bind polymerase tightly and give *slow* but detectable single-step additions. We would, however, predict that the two unassigned rapid-turnover additions (Table III) which are directed by a site or sites outside the early region are probably programmed by one or more of the minor promoters mapped there (Stahl & Chamberlin, 1977; Kassavetis et al., 1978). DNA sequences which are exceptions to the above trend plus investigations on how promoter sequence changes alter the rate of single-step additions should provide a useful insight into our concept of a "promoter" and its catalytic efficiency.

It seems likely that those combinations of substances which give rapid-turnover reactions should *not* be effective stabilizers of initiation complexes since the trinucleotide products dissociate so rapidly. Conversely, those combinations which are effective stabilizers would probably be synthesized at a very slow or negligible turnover rate. A comparison of our results with those of Dausse et al. (1975) seems to confirm that this is the case. For example, they reported that preincubation of RNA polymerase and T7 DNA with C-A and UTP results in some rifampicin- or poly(rI)-resistant synthesis of RNA, which was taken to be indicative of the formation of stable ternary initiation complexes. When the RNA products resulting from such synthesis were analyzed on agarose–acrylamide gels, products which had initiated at both the A1 and A3 promoters were found, indicating that C-A + UTP stabilized complexes at both of these promoters. There was considerably greater stabilization at the A3 promoter, however. Our results showed that C-A-U is produced about 10 times more rapidly at the A1 than at the A3 promoter. Thus, the reason that incubation with C-A + UTP is not very effective in stabilizing enzyme–DNA complexes at the A1 promoter is rapid dissociation of trinucleotide, not inefficient trinucleotide synthesis. Also, the combination of C-G + CTP which we found to give only a slow-turnover reaction at the A2 promoter was found by Dausse et al. (1975) to be quite effective in stabilizing RNA polymerase at this promoter. Finally, Minkley & Pribnow (1973) as well as Dausse et al. (1975) have reported that C-A + CTP stabilizes rifampicin-insensitive polymerase–DNA complexes at the A3 promoter, whereas we find that rapid C-A-C synthesis is not directed by the A3 promoter. The stabilization that they found could be due to the stoichiometric (or very slow) synthesis of C-A-C at A3 which we would not have detected. There is another possible mechanism in this case, however. C-A-C is synthesized rapidly elsewhere on the T7 DNA and could be present in the solution at micromolar or higher concentration. Thus, the binding of preformed C-A-C could be giving rise to the stabilization of enzyme–DNA complexes at A3. Experiments to investigate this possibility are currently being conducted.

References

Chamberlin, M. J. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 159–191, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Chamberlin, M. J., & Ring, J. (1972) *J. Mol. Biol. 70*, 221–237.

Darlix, J.-L., & Dausse, J.-P. (1975) *FEBS Lett. 50*, 214–218.

Dausse, J.-P., Sentenac, A., & Fromageot, P. (1975) *Eur. J. Biochem. 57*, 569–578.

Downey, K. M., Jurmark, B. S., & So, A. G. (1971) *Biochemistry 10*, 4970–4975.

Dunn, J. J., & Studier, F. W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1559–1563.

Hillel, Z., & Wu, C.-W. (1978) *Biochemistry 17*, 2954–2961.

Hoffman, D. J., & Niyogi, S. K. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 574–578.

Hsieh, T.-S., & Wang, J. C. (1976) *Biochemistry 15*, 5776–5783.

Johnston, D. E., & McClure, W. R. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 413–428, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Kassavetis, G. A., Kaya, K. M., & Chamberlin, M. J. (1978) *Biochemistry 17*, 5798–5804.
Lacks, S., & Greenberg, B. (1977) *J. Mol. Biol. 114*, 153–168.
Maniatis, T., Jeffrey, A., & Van de Sande, H. (1975) *Biochemistry 14*, 3787–3794.
Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560–564.
McClure, W. R., & Cech, C. L. (1978) *J. Biol. Chem. 253*, 8949–8956.
McClure, W. R., Cech, C. L., & Johnston, D. E. (1978) *J. Biol. Chem. 253*, 8941–8948.
McConnell, D. J. (1979) *Nucleic Acids Res. 6*, 525–544.
McDonell, M. W., Simon, M. N., & Studier, F. W. (1977) *J. Mol. Biol. 110*, 119–146.
Minkley, E. G. (1974) *J. Mol. Biol. 83*, 305–331.
Minkley, E. G., & Pribnow, D. (1973) *J. Mol. Biol. 77*, 255–277.
Oen, H., & Wu, C.-W. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1778–1782.
Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7*, 668–674.
Pribnow, D. (1975a) *Proc. Natl. Acad. Sci. U.S.A. 72*, 784–788.
Pribnow, D. (1975b) *J. Mol. Biol. 99*, 419–443.
Shapiro, A. L., Vinnela, E., & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun. 28*, 815–820.
Sharp, P. A., Sugden, B., & Sambrook, J. (1973) *Biochemistry 12*, 3055–3063.
Siebenlist, U. (1979) *Nucleic Acids Res. 6*, 1895–1907.
Stahl, S. J., & Chamberlin, M. J. (1977) *J. Mol. Biol. 112*, 577–601.
Studier, F. W. (1975) *J. Mol. Biol. 94*, 283–295.
Thomas, C. A., Jr., & Abelson, J. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G., & Davies, D., Eds.) Vol. I, pp 533–561, Harper and Row, New York.
Wu, C.-W., & Wu, F. Y.-H. (1978) *Biochemistry 17*, 138–144.

Peptidyl Transfer Ribonucleic Acid Hydrolase Activity of Proteinase *k*†

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ABSTRACT: Proteinase *k*, a seryl-protease obtained from *Tritirachium album*, is able to specifically hydrolyze N-blocked aminoacyl transfer ribonucleic acids (tRNAs). The blocked amino acid is released, and the tRNA molecule remains able to be recharged by its cognate amino acid. Aminoacyl-tRNAs are highly resistant to hydrolysis by the protease. This activity is not due to contamination of the protease preparation. A commercial protease from *Streptomyces griseus* displayed a similar activity, while trypsin, chymotrypsin, and papain unspecifically hydrolyzed all charged tRNAs tested. The characteristics of the hydrolysis performed by proteinase *k* closely resemble the peptidyl-tRNA hydrolase activity described in different cells as a scavenger for the peptidyl-tRNA that eventually falls from the polysomes. Our results warn about a hasty identification of any N-blocked aminoacyl-tRNA hydrolase activity in the cytoplasm as an independent peptidyl-tRNA hydrolase.

In previous experiments studying the sensitivity of ribosomal functions to proteases (Bernabeu et al., 1979), it was found that proteinase *k*, a protease obtained from *Tritirachium album* (Ebeling et al., 1974), was able to release ethyl acetate soluble radioactivity from the 3′-terminal fragment *N*-Ac-[^{3}H]Leu-ACCAC(U),[1] mimicking the "fragment reaction" catalyzed by ribosomal peptidyl transferase (Monro, 1971).

Although most proteases display a certain level of esterolytic activity, to our knowledge the sensitivity of the ester bond in the aminoacyl transfer ribonucleic acids (tRNAs) to the catalytic action of these enzymes has not been studied extensively before. Given the esterolytic activity of proteinase *k*, we considered it of interest to study in detail the characteristics of the reaction that takes place in the catalytic hydrolysis of aminoacyl-tRNA by proteases. The resulting data are reported here and show a surprising similarity between the specificity of substrate displayed by proteinase *k* and that of some peptidyl-tRNA hydrolases reported in the literature (Cuzin et al., 1967; Vogel et al., 1968; De Groot et al., 1969).

Materials and Methods

Labeled aminoacyl-tRNAs were obtained by charging commercial tRNA from *Escherichia coli* and yeast with the appropriate radioactive amino acids, using the homologous supernatant fraction (S-100) as a source of aminoacyl-tRNA synthetases. Separation of the [^{35}S]Met-tRNA$_f$ and [^{35}S]-Met-tRNA$_m$ was achieved on a column of BD-cellulose as described elsewhere (Carrasco et al., 1976). The 3′-terminal fragments were prepared from the charged aminoacyl-tRNA by treatment with RNase T_1 as described by Monro (1971). Proteinase *k* was obtained from Merck. All the other proteases were purchased from Sigma Chemical Co.

Activity Tests. Protease activity was tested on radioactive ribosomal proteins by measuring the loss of radioactivity precipitable by 5% trichloroacetic acid (Cl_3AcOH). The reaction was carried out in 50 μL of 30 mM PO_4^{3-}, pH 7.4,

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[1] Abbreviations used: *N*-Ac-Leu-A, *N*-acetylleucyladenosine; PMFS, phenylmethanesulfonyl fluoride.

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