Photochemical Cross-linking of Unmodified Acetylvalyl-tRNA to 16S RNA at the Ribosomal P Site†

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ABSTRACT: Acetylvalyl-, acetylphenylalanyl-, and formylmethionyl-tRNA which were derivatized at their 4-thiouridine residues with the photoaffinity label, *p*-azidophenacyl bromide, were nonenzymatically bound to salt-washed ribosomes. More than 90% of the binding was to the P site as judged by reactivity with puromycin. Subsequent irradiation (>310 nm) of the tRNA–ribosome complexes resulted in the covalent linking of only the acetylvalyl-tRNA to the 30S subunit. Attachment was solely to the 16S RNA with an efficiency of cross-linking of 13–15%. Covalent linking was 90% inhibited by prior treatment with puromycin, showing that the covalent linking reaction had taken place at the P site. Cross-linking required irradiation and mRNA but was not dependent on the presence of the photoaffinity probe in the tRNA. tRNAs whose 4-thiouridine had been modified with unreactive analogues of *p*-azidophenacyl bromide or unmodified acetylvalyl-tRNA exhibited the same cross-linking behavior as photoaffinity probe-modified acetylvalyl-tRNA. Furthermore, even acetylvalyl-tRNA whose 4-thiouridine had been removed by treatment with H_2O_2 was quantitatively as active as unmodified tRNA. These results provide the first demonstration of direct photochemical cross-linking of tRNA to ribosomes.

Transfer RNA molecules which have been modified with affinity or photoaffinity labels have proven to be very useful in delineating the tRNA-binding sites on ribosomes [for reviews, see Cooperman, 1976, 1978; Kuechler, 1976; Ofengand et al., 1977; Pellegrini and Cantor, 1977; Zamir, 1977; also, Czernilofsky et al., 1977; Johnson et al., 1977; Sonenberg et al., 1977; Yukioka et al., 1977]. Most of these studies employed analogues of peptidyl-tRNA to probe the peptidyltransferase center of the ribosome. We have previously described the preparation of *Escherichia coli* $tRNA^{Val}$ which had been modified by the incorporation of APA-Br[1] at its 4-thiouridine residue and its use in labeling both the P and A sites of *E. coli* ribosomes at regions other than the peptidyltransferase center (Schwartz and Ofengand, 1974; Schwartz et al., 1975; Ofengand et al., 1977).

Our earlier experiments on the photoaffinity labeling of the ribosomal P site were performed under conditions which were expected to result in binding of the tRNA exclusively to the P site (Schwartz and Ofengand, 1974). However, a definitive demonstration that this was indeed the case was not provided. We report here that acetylvalyl-$tRNA^{APA}$ bound to ribosomes was 90% reactive with puromycin and that prior reaction with puromycin blocked the covalent linking reaction. The extent and pattern of covalent attachment were the same as that reported earlier, thus establishing that the previous cross-linking was also to the ribosomal P site.

We also investigated the cross-linking capacity of AcPhe-$tRNA^{APA}$ and fMet-$tRNA^{APA}$ which had been nonenzymatically bound to the P site and of AcVal-tRNA which had been modified at the 4-thiouridine residue with several other photoaffinity probes. During the course of this study, it became clear that the observed cross-linking was not due to the presence of photoaffinity labels in the tRNA. Furthermore, the 4-thiouridine residue was not even involved, since chemical removal of this residue by treatment with H_2O_2 had no effect on the extent of cross-linking. It appears that the covalent attachment described in this paper and previously (Schwartz and Ofengand, 1974) was a result of direct photochemical cross-linking between the 16S ribosomal RNA and some part of the tRNA other than the 4-thiouridine residue.

Materials and Methods

Chemicals. *E. coli* $tRNA^{Val}_1$, $tRNA^{fMet}$, and two or three × 1 M NH_4Cl washed ribosomes were obtained or prepared as described previously (Schwartz and Ofengand, 1974). Tight couple ribosomes, prepared according to the procedure of Noll et al. (1973), were 20% active in P-site binding. *E. coli* $tRNA^{Phe}$ (1100 pmol of Phe acceptance/A_{260} unit), poly(U_2,G), and poly(U) were obtained from Boehringer, and tritiated amino acids were from Amersham/Searle. Aminoacyl-tRNA synthetases were either the unfractionated mixture described by Muench and Berg (1966) or were partially purified by DEAE-cellulose chromatography. PA-Br, APA-Br, and APAA-Br were obtained or prepared as described (Schwartz and Ofengand, 1974; Ofengand et al., 1977). Ethyl 4-iodo-2-diazoacetoacetate, $ICH_2COCN_2CO_2C_2H_5$, was the generous gift of Dr. Barry Cooperman and Ms. Arlene Minnella, University of Pennsylvania. $N^{5,10}$-methenyltetrahydrofolate was prepared from N^5-formyltetrahydrofolate (calcium leucovorin, Lederle) by treatment with acid under reducing conditions. Twenty-five milligrams was dissolved in 2.0 mL of 50 mM mercaptoethanol, 0.22 mL of 1 M HCl was added, and the mixture was warmed slightly to dissolve the precipitate. Conversion to the $N^{5,10}$-methenyl derivative took place at room temperature and was monitored by the increase in absorption at 355 nm. Complete reaction required 1–1.5 h. The concentration was calculated from the ϵ_{355} (pH 1) of 25 × 10^3 (Tabor and Wyngarden, 1959).

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[1] Abbreviations used are: APA, *p*-azidophenacyl; APA-Br, *p*-azidophenacyl bromide; AcVal or AcPhe-$tRNA^{APA}$, *N*-acetylvalyl or *N*-acetylphenylalanyl-tRNA derivatized at its 4-thiouridine residue with the *p*-azidophenacyl group; PA, phenacyl; PA-Br, phenacyl bromide; APAA, *p*-azidophenacyl acetate; APAA-Br, *p*-azidophenacyl bromoacetate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; fMet-tRNA, formylmethionyl-tRNA; ^{4}Srd, 4-thiouridine; Me_2SO, dimethyl sulfoxide; Tris, tris(hydroxymethyl)aminomethane; cmo^5U, uridine-5-oxyacetic acid.

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Acetylaminoacyl-tRNA. Aminoacylation of *E. coli* $tRNA^{Val}$ and $tRNA^{Phe}$ was performed as described previously (Ofengand et al., 1974) for *E. coli* synthetase but with omission of the other 19 unlabeled amino acids. $tRNA^{fMet}$ was aminoacylated similarly but with 0.1 M Hepes, pH 8, as buffer and 4 mM ATP. Formylation of $tRNA^{fMet}$ was carried out in the same reaction mixture by adding 80 μM $N^{5,10}$-methenyltetrahydrofolate, since excess transformylase was present in the enzyme fraction ($N^{5,10}$-methenyltetrahydrofolate spontaneously converts to N^{10}-formyltetrahydrofolate in the neutral reaction mixture). The aminoacylated tRNAs were isolated by phenol treatment and alcohol precipitation. Acetylation of Phe- and Val-tRNA was performed essentially as described by Haenni and Chapeville (1966). One milliliter of aminoacyl-tRNA (2–3 A_{260} units/mL) in 10 mM KOAc, pH 5, 10 mM $Mg(OAc)_2$ was mixed with 1 mL of 2 M KOAc, pH 5. At 0 °C, 0.05 mL of acetic anhydride was added at 7.5-min intervals for a total of 0.2 mL. One milliliter of water was added, followed by 8 mL of ethanol. After standing at 0 °C for 30 min, the precipitated tRNA was collected by centrifugation, drained, dissolved in water, and passed over Sephadex G-50 to remove residual salts and ATP. Assay for the extent of acetylation (or enzymatic formylation) was done by incubation at 37 °C with 25 mM $CuSO_4$–0.2 M Tris, pH 7.5, according to Schofield and Zamecnik (1968). The appropriate incubation time for maximal discrimination between N-blocked and unblocked aminoacyl-tRNA was used in each case, 3 min for Phe- and Met-tRNAs and 25 min for Val-tRNA. The $t_{1/2}$ values for the aminoacyl-tRNAs and their *N*-acetyl derivatives were independently determined and, in general, agreed with the values given by Schofield and Zamecnik (1968), except for Val-tRNA for which we found a $t_{1/2}$ of 4.9 min compared to the reported value of 3.0 min.

Derivatization of tRNAs. The PA, APA, and APAA derivatives of AcVal- and AcPhe-tRNA were prepared by reaction of the acetylaminoacyl-tRNAs according to previously published procedures (Schwartz and Ofengand, 1974; Ofengand et al., 1977), since the acetylaminoacyl groups were stable under the conditions employed. fMet-$tRNA^{APA}$ was prepared by first derivatizing the uncharged tRNAs as above, followed by enzymatic aminoacylation and formylation. In all cases, the extent of reaction was monitored by assay for the ability to form the $^4Srd_8$–Cyd_{13} cross-link upon irradiation at 350 nm (Schwartz and Ofengand, 1974; Ofengand et al., 1977) and was >90% complete. Confinement of the site of reaction to the Srd residue was shown by (a) the stoichiometric addition of [^{14}C]APA groups, (b) the blockage of incorporation by *prior* $^4Srd_8$–Cyd_{13} cross-linking, and (c) identification of [^{14}C]-APA-Srd as the only modified nucleoside in a complete digest of [^{14}C]APA-$tRNA^{Val}$ (Schwartz et al., 1975; Ofengand et al., 1977). AcVal-tRNA modified with ethyl 4-iodo-2-diazoacetoacetate was prepared by reaction of 1.2 A_{260} units (1.5 nmol) of AcVal-tRNA with 6.4 mM reagent in 1.4 mL of 91% Me_2SO, 7 mM KPO_4, pH 7.4. After 10 min at 23 °C, the solution was briefly dialyzed vs. water, and the tRNA precipitated with 0.2 M KOAc, pH 5, 67% ethanol; 82% reaction was obtained. Reaction of AcVal-tRNA with iodoacetamide was done similarly in 90% Me_2SO, 10 mM KPO_4, pH 7.4, 2.5 mM iodoacetamide, and 0.8 μM AcVal-tRNA. After 10 min at 23 °C, 12 mM mercaptoethanol was added to quench the reaction and the tRNA isolated as described above.

Removal of 4-Thiouridine from $tRNA^{Val}$. $tRNA^{Val}_1$ was treated with H_2O_2 by a modification of the procedure of Scheit (1968) and Shugart (1972). To 3.1 A_{260} units of $tRNA^{Val}_1$ (A_{337}/A_{260} = 0.021 in 10 mM Hepes, pH 7.5, 5 mM Mg^{2+}) in 0.7 mL of 0.1 M Tris, pH 8.0, 4.4 mM $Mg(OAc)_2$ at 23 °C was added 20 μL of 30% H_2O_2. The course of the reaction was followed in a spectrometer by the loss of the 4-thiouridine absorption peak at 337 mm. The reaction followed first-order kinetics with a $t_{1/2}$ of 9 min under the given conditions. After 1 h, the tRNA was isolated by precipitation with 2% KOAc, pH 5, 67% ethanol at 0 °C for 30 min. The drained precipitate was dissolved in water. The modified tRNA could accept 1380 pmol of valine per A_{260} unit compared to a value of 1540 pmol per A_{260} unit for the untreated starting material. The absorption spectrum of the untreated and modified tRNA (inset to Figure 5) was obtained on a Cary Model 15 spectrophotometer at 0–0.1 full scale.

Filter Binding Assay. Noncovalent and covalent binding of acyl[^{3}H]aminoacyl-tRNA to ribosomes was measured by Millipore filter adsorption in 50 mM Tris, pH 7.4, 50 mM KCl, 20 mM or 0.1 mM Mg^{2+}, respectively, as reported previously (Schwartz and Ofengand, 1974). The values for irradiated control mixtures lacking ribosomes were subtracted and were approximately equivalent to the values for unirradiated complete mixtures. The percent cross-linking was calculated as the ratio of filter-bound radioactivity in 0.1 mM Mg^{2+} to that bound at 20 mM Mg^{2+}, times 100.

Results

Binding and Covalent Attachment of Ac[^{3}H]Val-$tRNA^{APA}$ to the Ribosomal P Site. In our previous report (Schwartz and Ofengand, 1974), we presented indirect evidence that the Val-$tRNA^{APA}$ had been bound and cross-linked to the P site. In order to further ensure that attachment was indeed to the P site, Ac[^{3}H]Val-$tRNA^{APA}$ was prepared as an analogue of peptidyl-tRNA. As shown in Figure 1, irradiation of a noncovalent AcVal-$tRNA^{APA}$–ribosome complex resulted in covalent attachment of the tRNA exclusively to the 30S subunit. As before, the cross-linking was dependent on irradiation and on the presence of poly(U_2,G) as a synthetic mRNA. The efficiency of the cross-linking reaction was 13 ± 3%. In order to determine if cross-linking was to the 16S RNA or to 30S proteins, the Ac[^{3}H]Val-$tRNA^{APA}$–30S subunit covalent complex was dissociated with 0.5% sodium dodecyl sulfate and analyzed by sucrose gradient centrifugation (Figure 2). Almost all of the tRNA associated radioactivity migrated with the 16S RNA (94% for the experiment shown). These results were essentially the same as those obtained previously with Val-$tRNA^{APA}$ (Schwartz and Ofengand, 1974).

Reaction with puromycin was used to verify that the ribosome-bound AcVal-tRNA which was capable of being cross-linked was located in the P site. It was not sufficient to show that puromycin could release 85–90% of the nonenzymatically bound AcVal-tRNA (as was the case), since only 10–16% of the bound tRNA became cross-linked after irradiation. Rather, it was necessary to show that pretreatment with puromycin would abolish the covalent linking reaction. Two identical mixtures of Ac[^{3}H]Val-$tRNA^{APA}$ and ribosomes were first incubated in order to allow binding to occur, puromycin was then added to one reaction, and both mixtures were incubated an additional 15 min at 37 °C, irradiated as before, and centrifuged in a sucrose gradient. Since the tRNA was aminoacylated with radioactive valine, the disappearance of radioactivity from the 30S region of the gradient could be considered a measure of the ability to react with puromycin. As shown in Figure 3, 87% of the tRNA-associated radioactivity disappeared from the 30S peak as a result of the puromycin treatment. These experiments confirm our earlier conclusion that, on irradiation, Val-$tRNA^{APA}$ which is bound to the P site becomes covalently attached exclusively to the 16S RNA of the 30S subunit.

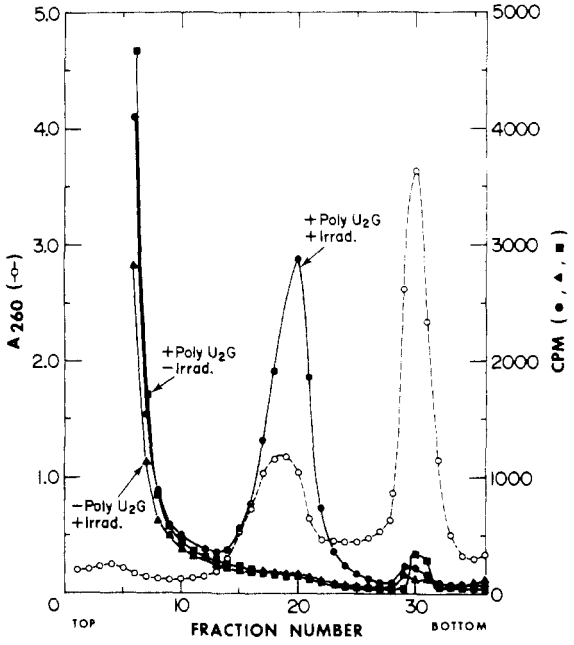

FIGURE 1: Covalent linking of AcVal-tRNA^{APA} exclusively to 30S subunits. The noncovalent binding of AcVal-tRNA^{APA} to ribosomes was carried out in 0.75-mL reaction mixtures containing 50 mM Tris, pH 7.4, 50 mM KCl, 20 mM $Mg(OAc)_2$, 5 mM DTT, 80 μg/mL poly(U_2,G), 50 A_{260} units/mL ribosomes, and 50 pmol/mL AcVal-tRNA^{APA}. The mixtures, minus tRNA, were incubated at 37 °C for 15 min to activate ribosomes (Zamir et al., 1974). After addition of tRNA, mixtures were incubated for an additional 15 min at 37 °C. Binding mixtures in Pyrex vessels were irradiated for 4 h at 0 °C in a Rayonet RPR-100 photochemical reactor equipped with 350-nm lamps as previously described (Schwartz & Ofengand, 1974; Ofengand et al., 1977). The mixtures were then dialyzed for 18 h at 0 °C against 10 mM Tris, pH 7.4, 50 mM NH_4Cl, 6 mM mercaptoethanol, and 0.3 mM $MgCl_2$ and layered on 36 mL of a 10–30% sucrose gradient made in the same buffer as the dialysate. Centrifugation was in an SW27 rotor at 24 000 rpm for 15 h. The A_{260} of the gradients were monitored in a Gilford 2400 spectrophotometer. Fractions of 1.0 mL were collected and the radioactivity was determined after the addition of 10 mL of Bray's solution. Noncovalent binding of the tRNA to ribosomes was determined by the filter-binding assay. For the complete system, 90 and 94% binding was obtained, before and after irradiation, respectively. Without poly(U_2,G), the values were 9 and 14%. The percent covalent linking was 14%, corrected for the minus irradiation or minus polynucleotide blank and adjusted for the percent noncovalent binding. (○) A_{260}; (●) complete incubation mixture; (▲) incubation mixture minus poly(U_2,G); (■) complete incubation mixture minus irradiation.

Cross-Linking of Other Derivatized tRNAs to the P site. Since all tRNAs are thought to have approximately the same molecular dimensions and all must pass through the common P site during protein synthesis, we tested the ability of two other representative tRNAs to be covalently linked to ribosomes. Both tRNAs chosen, tRNA^{Phe} and tRNA$^{Met}_f$, have a single 4-thiouridine in the same place as tRNA^{Val}, and consequently the APA probe should be in the same relative position both with respect to the tRNA and the ribosomal surface, when the tRNA is bound in the P site nonenzymatically. Surprisingly, however, only AcVal-tRNA^{APA} was effectively linked to the ribosome (Figure 4). Consequently, we decided to carefully examine other 4-thiouridine derivatives of tRNA^{Val} for their cross-linking activity in order to see what was unique about this tRNA.

Covalent Linking of Other Derivatives of AcVal-tRNA^{APA} to the P Site. Ac[^{3}H]Val-tRNA was prepared with two other photoaffinity labels attached to the 4-thiouridine residue. The first, APAA, was a 12-Å long phenylazide analogous to the 9-Å APA probe, and the second was ethyl 2-diazoacetoacetate, a photoreactive diazo ketone which generates a carbene 5 Å

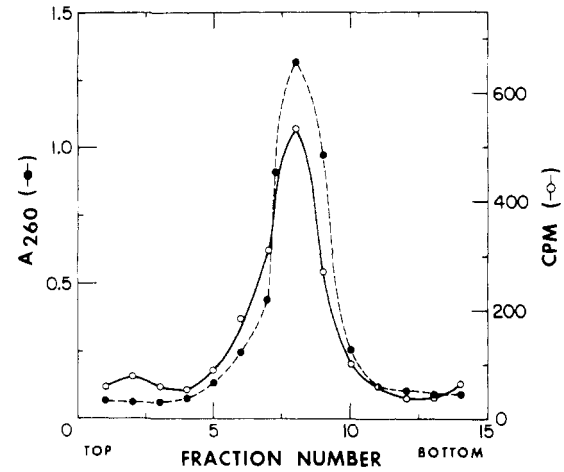

FIGURE 2: Distribution of covalently bound AcVal-tRNA^{APA} between 16S RNA and 30S ribosomal proteins. 30S subunit-AcVal-tRNA^{APA} covalent complexes were prepared by separation on sucrose gradients as described in the legend to Figure 3. The 30S-containing fractions were pooled, adjusted to 10 mM Mg^{2+}, and precipitated with KOAc/EtOH. The precipitate was redissolved in 10 mM sodium cacodylate, pH 5.8, 10 mM $MgCl_2$. Samples (150 μL) (containing 3600 cpm) were layered on 4 mL of a 5–20% sucrose gradient containing 20 mM sodium cacodylate, pH 5.8, 100 mM NaCl, 1 mM EDTA, and 0.5% sodium dodecyl sulfate and centrifuged in an SW 56 rotor at 50 000 rpm for 3 h. Fractions (0.25 mL) were analyzed for A_{260} and for acid-precipitable radioactivity. More than 86% of the recovered counts were found in the 16S RNA region of the gradient: (●) A_{260}; (○) cpm.

from the point of attachment. Ribosome-binding ability was unaffected by either of these modifications. Upon irradiation, both tRNAs became cross-linked to the ribosomal P site with the same efficiency as AcVal-tRNA (Table I). The result with the carbene probe was particularly surprising, since the carbene atom barely reaches to the periphery of the tRNA envelope using the yeast tRNA^{Phe} dimensions (Kim, 1976) as a model.

These data suggested that the observed cross-linking of modified Ac[^{3}H]Val-tRNA might not, in fact, be dependent on the presence of the photoaffinity label. This proved to be the case. When Ac[^{3}H]Val-tRNA^{PA} (modified at ^{4}Srd with phenacyl bromide which lacks the photolyzable azido group), Ac[^{3}H]Val-tRNA^{IAA} (modified at ^{4}Srd with iodoacetamide), or Ac[^{3}H]Val-tRNA (unmodified) were bound to the P site and irradiated, the tRNAs became covalently attached to ribosomes with efficiencies equivalent to those observed with the photoaffinity-label-modified tRNAs (Table I). Nevertheless, the cross-linking was dependent both on irradiation and the presence of a synthetic mRNA to direct binding to the P site. The 30S subunit-Ac[^{3}H]Val-tRNA^{APAA} covalent complex was further analyzed for distribution of the cross-linked tRNA between 16S RNA and ribosomal proteins and, as before, >90% of the radioactivity was associated with the 16S RNA.

Cross-Linking of 4-Thiouridine-Free AcVal-tRNA to Ribosomes. From the results described in the previous section, it was clear that chemically added probes were not a requirement for the cross-linking reaction. However, it was still possible that reaction had occurred at the ^{4}Srd locus, since the wavelengths of light used are known to activate ^{4}Srd (Frischauf and Scheit, 1973; Fiser et al., 1977), and the derivatives of ^{4}Srd which were used also absorb sufficiently in the >310-nm region to be potentially activatable. Accordingly, the ^{4}Srd was removed altogether by treatment with H_2O_2 which converts the ^{4}Srd to uridine (Scheit, 1968; Shugart, 1972). The inset in Figure 5 shows that this reaction resulted in complete disap-

TABLE I: Covalent Linking of Various Derivatives of AcVal-tRNA to the Ribosomal P Site.[a]

Srd derivative	Sp act. (pmol/A_{260} unit)	% covalently linked to 30S ribosome: Complete	Minus poly(U_2,G)	Minus irrad[b]	Assay method	Site of cross-link
$-CH_2CO$-Ph-N_3	1300	13 ± 3	<0.2	<0.2	Filter, gradient	RNA only
$-CH_2CO$-Ph-N_3	1300	*13.8	0.6		Gradient	
$-CH_2CO_2CH_2CO$-Ph-N_3	1250	15.8	0.3	<0.2	Gradient	RNA only
$-CH_2CO$-Ph	600	12.9	1.5		Gradient	
$-CH_2COCN_2CO_2Et$	1060	*12.3	0.6		Gradient	
$-CH_2CONH_2$	1390	13.5	<0.2		Filter	
None	1390	11.8	<0.2		Filter	

[a] Preparation of the various derivatives of AcVal-tRNA was carried out as described under Materials and Methods. The final acylation level for each preparation is given in the table. Incubation, irradiation, and analysis were performed essentially as described in the legend to Figure 3, with omission of the puromycin incubation, except that the ribosome concentration was 22 A_{260} units/mL and the tRNA was 40 pmol/mL. The two examples indicated by an asterisk were irradiated with the Rayonet 300-nm lamps without a Mylar filter for 1 h. (This condition gave maximal yield, as did the 4-h irradiation with 350-nm lamps.) The filter-binding assay was as described under Materials and Methods, and the sucrose gradient conditions were as in the legend to Figure 3. The percent covalent linking was calculated as the ratio of radioactivity in the 30S region to that in the entire gradient. Analysis of the distribution of covalent binding between RNA and protein was done as described in the legend to Figure 2. [b] Incubated in the dark for the same time as complete. In all cases, 0 time of irradiation gave blank (<0.2) values.

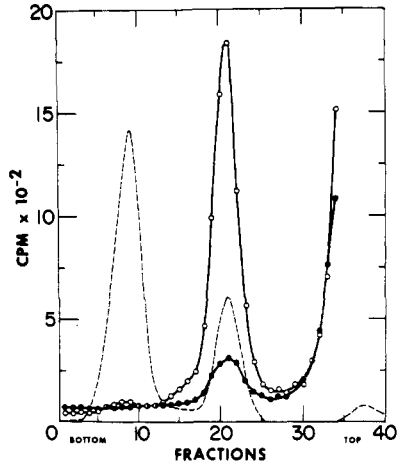

FIGURE 3: Puromycin sensitivity of the ribosome-bound AcVal-tRNA^{APA} capable of covalent cross-linking. Duplicate ribosome-binding mixtures of AcVal-tRNA^{APA} were incubated as described in the legend to Figure 1, except that the ribosome concentration was 42 A_{260} units/mL and the tRNA concentration was 32 pmol/mL. After the second incubation, puromycin was added to one binding mixture to a final concentration of 2.4 mM. Both mixtures were incubated for an additional 15 min at 37 °C and then irradiated as described in the legend to Figure 1. The samples were precipitated by the addition of 0.1 volume of KOAc, pH 5, and 2 volumes of EtOH, resuspended in 1.0 mL of 10 mM KPO_4, pH 6.2, 6 mM mercaptoethanol, and 0.25 mM $MgCl_2$, and layered on 10–30% sucrose gradients in the above sample buffer. Centrifugation and analysis were as in the legend to Figure 1. Covalent linking (12.6%) was obtained: (- - -) A_{260}; (○) complete reaction mixture minus puromycin; (●) complete reaction mixture plus puromycin.

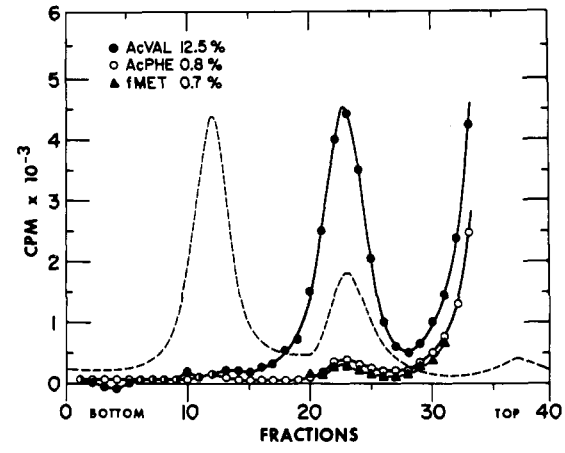

FIGURE 4: Covalent linking of AcVal-tRNA^{APA}, AcPhe-tRNA^{APA}, and fMet-tRNA^{APA} to the ribosomal P site. The noncovalent binding incubation for AcVal-tRNA^{APA} was carried out as described in the legend to Figure 3. The binding reaction for AcPhe-tRNA^{APA} was as described for AcVal-tRNA^{APA} with the following modifications: 10 mM $Mg(OAc)_2$, 0.1 mg/mL poly(U), 20 A_{260} units/mL ribosomes, 30 pmol/mL AcPhe-tRNA. The binding of fMet-tRNA^{APA} was performed in the absence of initiation factors as described for AcVal-tRNA^{APA} with the following modifications: 0.1 mg/mL poly(A,U,G), 100 A_{260} units/mL ribosomes, and 40 pmol/mL fMet-tRNA. Incubation, irradiation, isolation, and centrifugation were as described in the legend to Figure 3. Noncovalent binding was 81, 75, and 39% for AcVal-, AcPhe-, and fMet-tRNA, respectively. The corrected percent covalent binding is indicated on the figure: (●) AcVal-tRNA^{APA}; (○) AcPhe-tRNA^{APA}; (▲) fMet-tRNA^{APA}; (- - -) A_{260}.

pearance of the ^{4}Srd absorption band at 337 nm. The H_2O_2-treated tRNA^{Val} could be acylated with valine to 90% of the level of untreated tRNA and with approximately the same kinetics. Ribosomal P-site binding was the same as control tRNA. Irradiation of noncovalent complexes of ribosomes and H_2O_2-treated Ac[^{3}H]Val-tRNA resulted in covalent attachment of the tRNA to ribosomes. The kinetics of the cross-linking is depicted in Figure 5. It is clear that both control and H_2O_2-treated Ac[^{3}H]Val-tRNA link to ribosomes with identical kinetics and efficiency and that both require the presence of poly(U_2,G). Thus, it appears that unmodified Ac[^{3}H]Val-tRNA can be covalently attached to ribosomes by direct irradiation (>310 nm) and that this cross-linking is not dependent on the presence of ^{4}Srd in the tRNA. In this experiment, irradiation was with 300-nm lamps shielded by a sheet of Mylar polyester plastic to filter out light below 310 nm. However, the same results have also been obtained with either 350- or 300-nm lamps in the absence of the filter. Thus, control and H_2O_2-treated AcVal-tRNA show identical kinetics and yield of cross-linking independent of the spectral band used for irradiation.

In the experiment of Figure 5, the cross-linking efficiency (ca. 35%) was almost three times that reported in Table I. This is due to the use in the latter experiment of "tight couple" ribosomes prepared by the method of Noll et al. (1973). These ribosomes are four- to fivefold more active as judged by tRNA

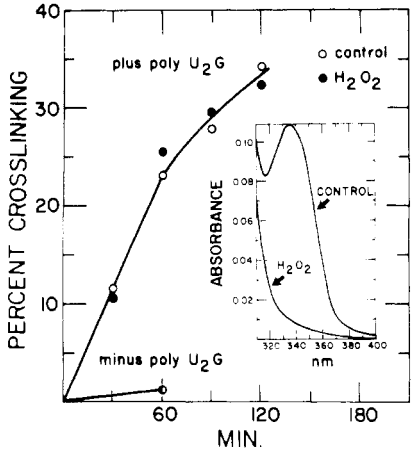

FIGURE 5: Kinetics of covalent linking of 4-thiouridine-less AcVal-tRNA to the ribosomal P site. Noncovalent binding to the ribosomal P site was carried out in mixtures containing 50 mM Hepes, pH 7.5, 50 mM NH_4Cl, 7 mM $MgCl_2$, 12 μg/mL poly(U_2,G), 5.6 A_{260} units/mL tight couple ribosomes, and 48 pmol/mL AcVal-tRNA (1.6-fold excess) for 5 min at 37 °C. (The binding reaction is actually complete in <0.5 min.) Sixty-two percent of the added tRNA was bound, and 21% of the ribosomes added was active. Irradiation was carried out at 0 °C in a Rayonet RPR-100 photochemical reactor equipped with nominal 300-nm (270–350 nm) lamps and a single sheet of Dupont Mylar S polyester plastic (92 gauge) to filter out light below 310 nm. The samples, in Pyrex tubes, were grouped at the center of the apparatus and continually stirred during irradiation. Covalent and noncovalent binding was measured by adsorption to nitrocellulose filters at 20 and 0.1 mM Mg^{2+}, respectively, as described under Materials and Methods. The 0 time value, corrected for the minus ribosome blank, was 0.4 and 0.2% for the control and H_2O_2-treated tRNA, respectively: (○) untreated AcVal-tRNA; (●) H_2O_2-treated AcVal-tRNA; (upper curve) plus poly(U_2,G); (lower curve) minus poly(U_2,G). Inset: absorbance spectrum in 10 mM Hepes pH 7.5, 5 mM Mg^{2+} of control and treated $tRNA^{Val}$ in the 4-thiouridine region of the spectrum: A_{260} of control, 5.20; A_{260} of H_2O_2-treated, 5.33.

binding and also allow greater levels of cross-linking. In more recent experiments, up to 60–70% of noncovalently bound unmodified AcVal-tRNA has been cross-linked, but still with exclusive attachment to 16S RNA. The increased efficiency cannot be attributed to the use of 300-nm lamps as in the experiment of Figure 5, since Table I (lines 1 and 2) as well as other experiments (manuscript in preparation) demonstrate that the same yield is obtained with either 300- or 350-nm lamps with or without the Mylar filter when a sufficient dose of radiation is used. Moreover, a direct comparison of tight-couple ribosomes vs. conventional salt-washed ribosomes showed an almost twofold increase in the extent of cross-linking when tight couples were used (manuscript in preparation).

Discussion

In our previous report of Val-$tRNA^{APA}$ cross-linking to the ribosomal P site (Schwartz and Ofengand, 1974), the presence of a large excess of ribosomes and the lack of tetracycline inhibition were taken as indirect evidence that the tRNA was found at the P site. This has now been confirmed directly by the fact that pretreatment with puromycin prevented covalent linking of ribosome-bound AcVal-tRNA (Figure 3). Moreover, in subsequent experiments (Ofengand, J., Liou, R., Kohut, J., Schwartz, I., and Zimmermann, R., manuscript in preparation), it was found that tRNA linked in a 70S-AcVal-tRNA covalent complex could still transfer 80–85% of its acetylvaline to puromycin. These results show that the tRNA is indeed attached to the ribosomal P site and that even after cross-linking the tRNA is still active in peptide-bond formation. We have also confirmed in this paper, using AcVal-tRNA, that the covalent attachment is exclusively (94%) to the 16S ribosomal RNA.

The findings presented here indicate that the cross-linking of AcVal-$tRNA^{APA}$ to 16S RNA is so far unique for this tRNA, since irradiation of P-site-bound AcPhe-$tRNA^{APA}$ or fMet-$tRNA^{APA}$ resulted in <1% cross-linking of these tRNAs to ribosomes (Figure 4). Initially, this was a surprising finding, since it would be assumed, a priori, that all tRNAs of the same structural class ($tRNA^{Val}$, $tRNA^{Met}_f$, and $tRNA^{Phe}$ are class I tRNAs) would exist in nearly identical conformations when bound to the ribosome and, therefore, would exhibit similar cross-linking properties. In this regard it should be noted that fMet-$tRNA^{APAA}$, bound to ribosomes in the presence of purified initiation factors, was cross-linked, specifically to 30S subunits, upon irradiation. The cross-linking was dependent on the presence of the photoaffinity probe in the tRNA and on irradiation (Schwartz et al., 1976).

The differences in the cross-linking behavior of the three P-site bound tRNAs can be explained by the data in Table I, which clearly indicate that the cross-linking, while requiring irradiation and mRNA, was not dependent on the presence of the photoaffinity probe in AcVal-tRNA. Indeed, the ^{4}Srd residue was not involved at all in the covalent attachment, since its removal from $tRNA^{Val}$ did not affect the cross-linking (Figure 5). It thus appears that the covalent linking of AcVal-tRNA to the 30S subunit is due to some other nucleotide(s).

Comparison of the sequences of the active and inactive tRNAs shows that the most obvious difference between the two classes is the presence of cmo^5U at the 5′-anticodon position in $tRNA^{Val}_1$. This residue, at the extreme tip of the tRNA molecule, is highly exposed and would be readily accessible to 16S RNA for the sort of close contact required for direct cross-linking. Tentative confirmation of this site of reaction has come from further studies with other tRNAs (Ofengand, J., Liou, R., Kohut, J., Schwartz, I. and Zimmerman, R., manuscript in preparation). *E. coli* valine tRNAs which lack cmo^5U, such as $tRNA^{Val}_2$ with a G replacing cmo^5U or 5-fluorouracil-substituted *E. coli* $tRNA^{Val}_1$ in which 5-fluorouracil replaces the uracil-5-oxyacetic acid residue, are inactive, while *E. coli* $tRNA^{Ser}_1$ which has cmo^5U at the same site as $tRNA^{Val}_1$ can be cross-linked about as well as $tRNA^{Val}_1$.

To our knowledge, this is the first report of direct photochemical cross-linking of tRNA to ribosomes. This approach has been used successfully to investigate aminoacyl-tRNA synthetase–tRNA interactions (Schoemaker and Schimmel, 1974; Budzik et al., 1975; Schoemaker et al., 1975), aminoacyl-tRNA synthetase–ATP interactions (Yue and Schimmel, 1977), mRNA–ribosome interactions (Fiser, et al., 1975, 1977; Schenkman et al., 1974), ribosomal protein–ribosomal RNA interactions (Gorelic, 1976a,b; Möller and Brimacombe, 1975; Rinke et al., 1976; Ehresmann et al., 1975, 1976, 1977; Baca and Bodley, 1976), EFG–GTP interaction (Rohrbach and Bodley, 1977), and ribosome–antibiotic interaction (Jaynes et al., 1977; Cooperman et al., 1975; Sonenberg et al., 1974). The two main advantages of direct photochemical cross-linking of unmodified ligands to their receptors are the elimination of possible perturbations of the natural complex by introduction of an extraneous chemical moiety in the ligand and the fact that the probe is of zero length; that is, the two partners in the reaction must be within covalent bonding distance of each other for cross-linking to occur. At the same time, however, the use of unmodified ligands frequently requires the use of short-wavelength UV irradiation with its attendant photoactivation of unwanted regions of the macromolecules under study and

consequent inactivation. In some cases, the problem can be overcome by acetone photosensitization (see, for example, Sperling and Havron, 1976), and in others (Fiser et al., 1977; Sonenberg et al., 1974), and this work, the natural ligands used can be activated at sufficiently long wavelengths so as to minimize the problem. The cross-linked tRNA and ribosomes were still functionally active, as evidenced by their reactivity with puromycin (cited above), and prephotolysis of both tRNA and ribosomes did not affect either their subsequent binding or cross-linking ability (manuscript in preparation).

In light of the experiments presented here, the cross-linking of Val-tRNA^{APA} to the ribosomal A site was reevaluated. We previously reported (Schwartz, et al., 1975) that Val-tRNA^{APA}, enzymatically bound to the A site in the presence of EFTu, became covalently attached in a reaction which was dependent on irradiation and the presence of the APA probe in the tRNA. These results were confirmed and extended to include Phe-tRNA^{APA}, a tRNA which does not cross-link at all to the ribosome when bound in the P site but which becomes cross-linked to the A site in an EFTu, poly(U), irradiation, and APA-dependent manner (unpublished results). These contrasting findings underline the structural distinction between P and A sites that we have pointed out earlier (Schwartz et al., 1975).

The most striking finding of this work is the existence of an extremely close contact between tRNA (probably at the 5′-anticodon base) and 16S RNA. This only occurs when tRNA occupies the P site. Our previous studies (Schwartz et al., 1975) showed that Val-tRNA^{PA} bound at the A site did not cross-link and this has been recently confirmed using unmodified Val-tRNA (Hsu and Ofengand, unpublished results). The contact must be quite close, since up to 70% covalent linking has been obtained in some experiments (35% in Figure 5). Perhaps 16S RNA is involved in the anticodon conformational change postulated by Kim (1978) to occur upon translocation from the A to the P site.

The point of attachment to 16S RNA has been partially characterized. It is within the 3′-third of the 16S RNA but not within the colicin E3 fragment, and there is no indication for more than one site of attachment to the 16S RNA (Zimmermann, R., Gates, S., Schwartz, I., and Ofengand, J., manuscript in preparation).

The fact that covalent linking is mRNA-dependent implies the existence of a codon–anticodon interaction at the same time as the 5′-anticodon base is interacting with or is near to the 16S RNA. This could be a sterically complex situation. However, as there is now accumulating evidence that the third letter of the codon, corresponding to the 5′-anticodon base, may not be used at all or may be read in unexpected ways (Mitra et al., 1977; Weissenbach et al., 1977), simultaneous interaction of the anticodon with mRNA and 16S RNA may not be a problem. The exact structure of the covalent link between the tRNA and 16S RNA would be very helpful in understanding the spatial relationship at the decoding P site of the ribosome and such studies are currently in progress.

References

Baca, O. G., and Bodley, J. W. (1976), *Biochem. Biophys. Res. Commun. 70,* 1091–1096.

Budzik, G. P., Lam, S. S. M., Schoemaker, H. J. P., and Schimmel, P. R. (1975), *J. Biol. Chem. 250,* 4433–4439.

Cooperman, B. (1976), in Aging, Carcinogenesis and Radiation Biology, Smith, K. C., Ed., New York, N.Y., Plenum Press, pp 315–340.

Cooperman, B. S. (1978), in Bioorganic Chemistry, Vol. 4, van Tamelen, E., Ed., New York, N.Y., Academic Press, pp 81–115.

Cooperman, B. S., Jaynes, E. N., Brunswick, D. J., and Luddy, M. A. (1975), *Proc. Natl. Acad. Sci. 72,* 2974–2978.

Czernilofsky, A. P., Collatz, E., Gressner, A. M., and Wool, I. G. (1977), *Mol. Gen. Genet. 153,* 231–235.

Ehresmann, B., Reinbolt, J., and Ebel, J. P. (1975), *FEBS Lett. 58,* 106–111.

Ehresmann, B., Reinbolt, J., Backendorf, C., Tritsch, D., and Ebel, J. P. (1976), *FEBS Lett. 67,* 316–319.

Ehresmann, B., Backendorf, C., Ehresmann, C., and Ebel, J. P. (1977), *FEBS Lett. 78,* 261–266.

Fiser, I., Margaritella, P., and Kuechler, E. (1975), *FEBS Lett. 52,* 281–283.

Fiser, I., Scheit, K. H., and Kuechler, E. (1977), *Eur. J. Biochem. 74,* 447–456.

Frischauf, A. M., and Scheit, K. H. (1973), *Biochem. Biophys. Res. Commun. 53,* 1227–1233.

Gorelic, L. (1976a), *Biochemistry 15,* 3579–3590.

Gorelic, L. (1976b), *Biochim. Biophys. Acta 454,* 185–192.

Haenni, A. L., and Chapeville, F. (1966), *Biochim. Biophys. Acta 114,* 135–148.

Jaynes, E. N., Jr., Grant, P. G., Giangrande, G., Wieder, W., and Cooperman, B. S., (1978), *Biochemistry 17,* 561–569.

Johnson, A., Fairclough, R., and Cantor, C. (1977), in Nucleic Acid-Protein Recognition, Vogel, H. J., Ed., New York, N.Y., Academic Press, pp 469–490.

Kim, S. H. (1976), *Progr. Nucleic Acid Res. Mol. Biol. 17,* 181–216.

Kim, S. H. (1978), in Transfer RNA, S. Altman, Ed., Cambridge, Mass., The M.I.T. Press (in press).

Kuechler, E. (1976) *Angew. Chem., Int. Ed. Engl. 15,* 533–542.

Mitra, S. K., Lustig, F., Akesson, B., and Lagerkvist (1977), *J. Biol. Chem. 272,* 471–478.

Möller, K., and Brimacombe, R. (1975), *Mol. Gen. Genet. 141,* 343–355.

Muench, K., and Berg, P. (1966), *Proced. Nucleic Acid Res. 1,* 375–383.

Noll, M., Hapke, B., Schreier, M. H., and Noll, H. (1973), *J. Mol. Biol. 75,* 281–294.

Ofengand, J., Chladek, S., Robilard, G., and Bierbaum, J. (1974), *Biochemistry 13,* 5425–5432.

Ofengand, J., Schwartz, I., Chinali, G., Hixson, S. S., and Hixson, S. H. (1977), *Methods Enzymol. 46,* 683–702.

Pellegrini, M., and Cantor, C. (1977), in Molecular Mechanisms of Protein Biosynthesis, Weissbach, H., and Pestka, S., Ed., New York, N.Y., Academic Press, pp 203–244.

Rinke, J., Yuki, A., and Brimacombe, R., (1976), *Eur. J. Biochem. 64,* 77–89.

Rohrbach, M. S., and Bodley, J. W. (1977), *Arch. Biochem. Biophys. 183,* 340–346.

Scheit, K. H. (1968), *Biochim. Biophys. Acta 166,* 285–293.

Schenkman, M. L., Ward, D. C., and Moore, P. B., (1974), *Biochim. Biophys. Acta 353,* 503–508.

Schoemaker, H. J. P., and Schimmel, P. R. (1974), *J. Mol. Biol. 84,* 503–513.

Schoemaker, H. J. P., Budzik, G. P., Giege, R., and Schimmel P. R., (1975), *J. Biol. Chem. 250,* 4440–4444.

Schofield, P., and Zamecnik, P. C. (1968), *Biochim. Biophys. Acta 155,* 410–416.

Schwartz, I., and Ofengand, J. (1974), *Proc. Natl. Acad. Sci. 71,* 3951–3955.

Schwartz, I., Gordon, E., and Ofengand, J. (1975), *Bio-*

chemistry 14, 2907-2914.
Schwartz, I., Tejwani, R., and Ofengand, J. (1976), *Proc. Int. Congr. Biochem., 10th,* Hamburg, Abstr. 03-3-154.
Shugart, L. (1972), *Arch. Biochem. Biophys. 148,* 488-495.
Sonenberg, N., Zamir, A., and Wilchek, M. (1974), *Biochem. Biophys. Res. Commun. 59,* 693-696.
Sonenberg, N., Wilchek, M., and Zamir, A. (1977), *Eur. J. Biochem. 77,* 217-222.
Sperling, J., and Havron, A. (1976), *Biochemistry 15,* 1489-1495.
Tabor, H., and Wyngarden, L. (1959), *J. Biol. Chem. 234,* 1830-1846.
Weissenbach, J., Dirheimer, G., Falcoff, R., Sanceau, J., and Falcoff, E. (1977), *FEBS Lett. 82,* 71-76.
Yue, V. T., and Schimmel, P. R. (1977), *Biochemistry 16,* 4678-4684.
Yukioka, M., Hatayama, T., and Omori, K. (1977), *Eur. J. Biochem. 73,* 449-459.
Zamir, A. (1977), *Methods Enzymol. 46,* 621-637.
Zamir, A., Miskin, R., Vogel, F., and Elson, D. (1974), *Methods Enzymol. 30,* 406-426.

Initiation of Enzymatic DNA Synthesis by Yeast RNA Polymerase I†

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ABSTRACT: In vitro DNA synthesis by yeast DNA polymerase I can be initiated by partially purified yeast RNA polymerases in the presence or absence of rNTPs. Homogeneous yeast RNA polymerase I initiates DNA synthesis by yeast DNA polymerase I on single-stranded DNA templates only in the presence of all four rNTPs. A protein capable of initiating enzymatic DNA synthesis on single-stranded DNA in the absence of rNTPs has also been separated from partially purified yeast RNA polymerase I fractions. Analyses of the RNA polymerase I initiated replication products of phage fd DNA on alkaline sucrose gradients showed noncovalent linkage between the newly synthesized DNA and the template. Isopycnic analyses of the ribonucleotide initiated fd DNA replication products demonstrated covalent linkage between the initiator RNA and newly synthesized DNA. Results from ^{32}P-transfer experiments confirmed the covalent linkage between RNA and DNA chains and showed the presence of all four ribo- and deoxyribonucleotides at the RNA-DNA junctions. The ribonucleotide found most frequently at the RNA-DNA junction is uridylate and the purine deoxynucleotides occur more frequently than pyrimidine deoxynucleotides.

Purified DNA polymerases from prokaryotic and eukaryotic cells cannot initiate DNA chains de novo (Kornberg & Kornberg, 1974; Bollum, 1974). Analyses of newly synthesized DNA chains in several eukaryotic systems showed the presence of RNA at the 5′ end (Reichard et al., 1974; Waqar & Huberman, 1975a,b; Tseng et al., 1975). It is not known, however, whether all newly synthesized DNA chains are initiated with RNA primers or what enzyme(s) in eukaryotic systems might be involved in synthesis of the primers used in DNA synthesis.

Two DNA polymerases are present in yeast extracts (Wintersberger & Wintersberger, 1970; Helfman, 1973). Studies using synthetic template systems showed that only DNA polymerase I will use oligoribonucleotide as an initiator for the replication of a polydeoxynucleotide (Chang, 1977). In a search for enzymes or proteins capable of initiating DNA synthesis in vitro, we reported that all three partially purified yeast RNA polymerases are capable of initiating DNA polymerase I catalyzed DNA replication in the absence of rNTPs (Plevani & Chang, 1977). The initiation can be enhanced by the addition of all four rNTPs in the in vitro system.

To examine the apparent duality of the partially purified RNA polymerase in the initiation of enzymatic DNA synthesis in the presence and in the absence of rNTPs, it was necessary to continue the purification of the RNA polymerase. We therefore purified yeast RNA polymerase I to homogeneity (Valenzuela et al., 1976) and followed the substrate requirements for initiation activities. This communication describes the separation of rNTP dependent initiation activity of yeast RNA polymerase from a separate component that appears to initiate DNA synthesis with dNTPs alone. The products of yeast RNA polymerase I initiated DNA synthesis and the properties of the RNA polymerase-DNA polymerase coupled reactions are also characterized.

Experimental Procedure

Chemicals Substrates and DNAs. Deoxynucleotide triphosphates (dNTPs)[1] were prepared as previously described (Chang & Bollum, 1971). Commercial sources of other materials used were: rNTPs, [*methyl*-^3H]dTTP, and [2-^{14}C]ATP from Schwarz/Mann; [α-^{32}P]dNTPs from New England Nuclear; Qβ RNA, fd DNA, and $\phi\chi$ 174 DNA from Miles Laboratories; calf thymus DNA and bovine serum albumin from Worthington Biochemical Corp. Phage T_7 DNA was a generous gift from Dr. B. Alberts, University of California at

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[1] Abbreviations used for nucleotides are those recommended by the Commission on Biochemical Nomenclature (CBN) of IUPAC-IUB as approved by the Commission of Editors of Biochemical Journals [(1970) *Biochemistry, 9,* 4022]. Other abbreviations used: DTT, dithiothreitol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetate.

0006-2960/78/0417-2530$01.00/0