DNA-DEPENDENT RNA-DIRECTED PROTEIN SYNTHESIS IN VITRO, I. EXTENT OF GENOME TRANSCRIPTION*

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Abstract and Summary.—We have described an *in vitro* system which couples UTP incorporation into RNA by DNA-dependent RNA polymerase with RNA-directed amino acid incorporation into polypeptides. Using the technique of DNA-RNA hybridization, we have demonstrated that the whole Φ X-174 genome is transcribed during concomitant protein synthesis.

We are studying an *in vitro* system which couples transcription to translation. The replicative form of DNA (RF-DNA) from the bacteriophage Φ X-174 directs RNA synthesis by RNA polymerase which is coupled to protein synthesis with ribosomes and soluble components extracted from *E. coli* Q13. Our goal in developing this system is to mimic the *in vivo* transfer of genetic information from DNA to protein.

One question that we asked of the coupled system was the extent of genome transcription. We approached the answer by the following argument. If we had an RNA that was complementary to the whole genome, the saturation plateau generated by hybridizing increasing amounts of the RNA to a fixed amount of RF-DNA would represent 100 per cent transcription. RNA extracted from the coupled system (coupled RNA) could be hybridized to RF-DNA, and a comparison of the saturation plateaus would indicate the extent of transcription.

It is possible to synthesize an RNA that is complementary to most of the genome, the RNA transcribed from the single-stranded DNA of the mature virus (Φ X-DNA). The transcription product is a DNA-RNA hybrid.¹⁻³ The extent of the reaction can be measured by the shift in the buoyant density, and the density reaches a limiting value when the ratio of DNA to RNA in the hybrid is 1:1. Free RNA does not appear in the reaction mixture until the limiting density is reached. The hybrid is a double-stranded circle as revealed by electron microscopy, and its circumference is very close to that of RF-DNA³ (also a double-stranded circle⁴). X-ray diffraction data from a similar single-stranded DNA phage DNA-RNA hybrid show that the hybrid is a hydrogen-bonded structure of helical conformation.⁵ Since the hybrid possesses a definite density, it is possible to monitor the reaction and extract RNA which is complementary to most of the genome. We call this noncoding RNA since it is synthesized on the noncoding strand of RF-DNA.⁶

In this paper we describe hybridization experiments showing that the whole Φ X-174 genome is transcribed during protein synthesis in the coupled system.

Materials and Methods.—*Purification of Φ X-174 DNA and RF-DNA* was reported previously.⁷ The 21S form of RF-DNA⁴ was used exclusively, except for the preparation of DNA filters for hybridization, in which case 16S heat-denatured RF-DNA was used.⁸

DNA-dependent RNA polymerase was isolated from frozen *E. coli* C BTCC122 (Φ X-174 host) by a modification²² of the procedure of Chamberlin and Berg.⁹ Initial activity was 2580 units per milligram.

Noncoding RNA was synthesized by the method of Chamberlin and Berg.⁹ The reaction mixture contained (per milliliter and in order of addition): H_2O , 40 μmoles tris-HCl, pH 7.9, 1 μmole MnCl_2 , 4 μmoles MgCl_2 , 12 μmoles 2-MSH, 0.5 μmole each of ATP, GTP, CTP, and H^3 -UTP, 25–100 μg $\Phi\text{X-174}$ DNA, and 100 μg RNA polymerase. After incubation at 37°C the reaction was stopped by addition of phenol, and the nucleic acids were purified by phenol extraction followed by ether treatment.¹⁰ After extensive dialysis against $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl, 0.015 M Na citrate), a small portion of the product was taken for analysis in the analytical ultracentrifuge. The remainder was dialyzed against TM buffer ($3 \times 10^{-2} M$ tris-HCl, $5 \times 10^{-3} M$ MgCl_2 , pH 7.2–7.4) overnight. The dialyzed solution was heated for 10 min at 95°C and rapidly chilled to 0°C. Electrophoretically pure DNase (General Biochemicals) was added to a final concentration of 10 $\mu\text{g}/\text{ml}$, and the mixture was incubated at 37°C for 20 min. The enzyme was inactivated by heating for 10 min at 80°C, and the RNA, now free of DNA, was dialyzed against $2 \times \text{SSC}$.

Isopycnic centrifugation in Cs_2SO_4 was used to determine the buoyant density of the DNA-RNA hybrid formed during the synthesis of noncoding RNA. The sample, 0.80 ml of $10^{-2} M$ tris-HCl, pH 7.9, contained 8 μg of the nucleic acid preparation and 2 μg $\Phi\text{X-174}$ DNA as marker ($\rho = 1.456 \text{ gm/cc}$).² Cs_2SO_4 (Harshaw Chemical Co.) was added to a final concentration of approximately 44% (w/w) ($\rho = 1.533 \text{ gm/cc}$), and the sample was centrifuged at 44,770 rpm at 25°C in a Beckman model E analytical ultracentrifuge. After 18 hr the sample was photographed with UV illumination, and the negative was traced with a Joyce-Loebl microdensitometer. The buoyant density of the hybrid was calculated² to be 1.504 gm/cc which agrees with the published value.^{2, 3}

Coupled system extracts were prepared from frozen *E. coli* Q13 (RNase I⁻, polynucleotide phosphorylase⁻) by a modification of the method of Nirenberg and Matthaei¹¹ in which DNase treatment was omitted. All operations were performed at 0–4°C; 8-gm cells in early log phase were disrupted in a French press with 8 ml TMK buffer ($10^{-2} M$ tris-HCl, $10^{-2} M$ $\text{Mg}(\text{OCOCH}_3)_2$, $6 \times 10^{-2} M$ KCl, $6 \times 10^{-2} M$ 2-MSH, pH 7.7–7.8). The resulting suspension was centrifuged 20 min at $20,000 \times g$, and the supernatant was centrifuged 30 min at $30,000 \times g$. The following components were added to the top three fourths of the $30,000 \times g$ supernatant (in order of addition and to a final concentration of): $4 \times 10^{-2} M$ tris-HCl, $12 \times 10^{-3} M$ PEP, $4 \times 10^{-6} M$ each of 20 amino acids, $9 \times 10^{-3} M$ 2-MSH, $25 \times 10^{-4} M$ ATP, $5 \times 10^{-4} M$ GTP, and 50 $\mu\text{g}/\text{ml}$ pyruvate kinase (Calbiochem, 251 units/mg). The mixture was incubated 80–90 min at 33°C to eliminate endogenous mRNA and dialyzed 10–14 hr against three changes of TMA 0.06 buffer ($10^{-2} M$ tris-HCl, $10^{-2} M$ $\text{Mg}(\text{OCOCH}_3)_2$, $6 \times 10^{-2} M$ NH_4Cl , $6 \times 10^{-2} M$ 2-MSH, pH 7.7–7.8). The dialyzed solution was centrifuged 10 min at $30,000 \times g$, and ribosomes were precipitated from the supernatant by centrifugation for 2 hr at $105,000 \times g$ in the no. 40 rotor of the Spinco L2 preparative ultracentrifuge. The high-speed supernatant was centrifuged again for 10 hr at $105,000 \times g$. At the end of the run, the top 3 ml were discarded, and the next 9 ml were stored at -90°C as the soluble fraction ($\text{OD}_{260 \text{ m}\mu} = 25/\text{ml}$). The ribosomal pellet was washed with a small volume of TMA 0.3 buffer (same as TMA 0.06, but NH_4Cl concentration is 0.3 M), resuspended in that buffer, and stored at 0°C overnight. The suspension was centrifuged 10 min at $30,000 \times g$, the pellets were discarded and the ribosomes were precipitated from the supernatant by centrifugation for 90 min at $105,000 \times g$. The pellet was washed in TMA 0.06 buffer, resuspended, and centrifuged 10 min at $30,000 \times g$ as before. The ribosomal fraction ($\text{OD}_{260 \text{ m}\mu} = 500/\text{ml}$) was stored at -90°C .

RNA and protein synthesis in the coupled system were measured in a standard reaction mixture containing 1.65 μmoles tris-HCl, pH 7.8, 9.0 μmoles NH_4Cl , 0.5 μmole $(\text{NH}_4)_2\text{SO}_4$, 0.1 μmole NaCl, 1.5 μmole $\text{Mg}(\text{OCOCH}_3)_2$, $5 \times 10^{-3} \mu\text{moles}$ MgCl_2 , 0.9 μmole 2-MSH, $25 \times 10^{-3} \mu\text{moles}$ GSH, 0.6 μmole PEP, 0.25 μmole ATP, 0.05 μmole each of GTP, CTP, and H^3 -UTP, $4 \times 10^{-3} \mu\text{moles}$ each of 20 amino acids (C^{14} -valine), 5 μg pyruvate kinase, 2–5 μg RF-DNA, 12.5 μg RNA polymerase, 5 $\text{OD}_{260 \text{ m}\mu}$ units ribosomal fraction, and 0.75 $\text{OD}_{260 \text{ m}\mu}$ unit soluble fraction, in a final volume of 0.13 ml. The RF-

DNA buffer (10^{-2} M tris-HCl, 10^{-2} M NaCl, pH 7.3) contributed 0.1 μ mole tris-HCl and all the NaCl. The RNA polymerase buffer (10^{-2} M tris-HCl, 10^{-3} M MgCl_2 , 5×10^{-3} M GSH, 0.1 M $(\text{NH}_4)_2\text{SO}_4$, pH 7.6) contributed 0.05 μ mole tris-HCl and all the MgCl_2 , GSH, and $(\text{NH}_4)_2\text{SO}_4$.

After incubation at 33°C the SRM was mixed with 1.87 ml of a 1% casamino acid solution; 1.0 ml of the mixture was brought to a final TCA concentration of 5%, and the precipitate was collected on nitrocellulose membrane filters. After the filters were dried, they were counted in a liquid scintillation counter. This fraction (cold TCA-precipitable counts) gave the UTP incorporation into RNA. The other half of the reaction mixture was brought to a final PCA concentration of 7% and incubated for 20 min at 90°C ; 0.05 ml of 5 mg/ml bovine serum albumin was added and the precipitate collected by centrifugation. The pellet was dissolved in 0.2 N NaOH and the solution brought to a final TCA concentration of 5%. The precipitate was plated and counted as before. This fraction (hot PCA-insoluble counts) gave the incorporation of amino acids into protein.

Coupled and uncoupled RNA were synthesized in a $10\text{--}20 \times$ SRM. "Uncoupled" RNA was synthesized by omitting ribosomes and soluble fraction. After incubation at 33°C for 5 or 30 min the reaction was stopped by chilling to 0°C ; $0.5 \times$ SRM was removed to measure RNA and protein synthesis, and the remaining nucleic acids were extracted with phenol followed by ether treatment.¹⁰ To the nucleic acid solution, 0.1 vol of 3 M sodium acetate was added and the nucleic acids were precipitated with 3 vol of absolute ethanol. The precipitate was dissolved in TM buffer, and RNA was freed of DNA as described for *noncoding RNA*.

Hybridizations were performed by the method of Gillespie and Spiegelman.¹² Adsorption of RNA to blank filters was reduced by using filters 10 or 12 mm in diameter and carrying out the hybridization in 0.1–0.2 ml. 16S RF-DNA was heat-denatured imme-

FIG. 1.—Kinetics of $\text{H}^3\text{-UTP}$ and $\text{C}^{14}\text{-valine}$ incorporation in coupled system (extract B-193). A standard reaction mixture is described in *Materials and Methods*. ($\text{H}^3\text{-UMP} + \text{RF-DNA}$ $\bigcirc\text{--}\bigcirc$ or $-\text{RF-DNA}$ $\bigcirc\text{--}\bigcirc$; $\text{C}^{14}\text{-valine} + \text{RF-DNA}$ $\bullet\text{--}\bullet$ or $-\text{RF-DNA}$ $\bullet\text{--}\bullet$).

diately before use.⁸ Similar results were obtained by fixing RF-DNA to the filters by filtration or by spotting with a 10- μ l drop. After hybridization the filters were treated with an RNase solution containing 30 μ g/ml of five-times recrystallized bovine pancreatic RNase (Sigma; heat-treated as previously described⁵) and 15 units/ml of RNase T₁ (Sankyo Co., Tokyo, Japan) for 60 min at 25°C, and then they were washed with an excess of 2 $\times$ SSC.

Sucrose gradients were prepared in TS 0.05 buffer (3×10^{-2} M tris-HCl, 5×10^{-2} M NaCl, pH 7.2–7.4), 5–20% (w/w) sucrose. *E. coli* P³²-RNA was added as marker. Samples were centrifuged in the Spinco SW50 rotor for 275 min at 5°C, and drops were collected on pieces of Whatman 3 MM filter paper. Filter papers were washed in cold 5% TCA and 95% ethanol, dried, and counted.

TABLE 1. *Inhibition of amino acid incorporation and RNA synthesis.**

Treatment	Hot PCA-insoluble C ¹⁴ -valine incorporation (μ moles/SRM)	Cold TCA-insoluble H ³ -UTP incorporation (μ moles/SRM)
1. Complete†	98.2	3100
2. – 19 amino acids‡	5.3	3200
3. – ribosomes	0.1	3065
4. – soluble fraction	0.2	2612
5. – RF-DNA	0.3	450
6. + DNase, 1 μ g	0.1	10
7. – polymerase	3.4	145
8. + RNase, 0.5 μ g	0.4	12
9. + actinomycin, 1 μ g	0.2	30

* The reaction was carried out at 33°C for 1 hr (extract CS-8).

† The complete reaction mixture is described in the text.

‡ Only C¹⁴-valine exists in the reaction mixture.

Results and Discussion.—Figure 1 illustrates the kinetics of the coupled system. H³-UTP is incorporated into RNA for 30 minutes, and C¹⁴-valine is incorporated into peptides for at least 60 minutes after an initial lag. C¹⁴-valine incorporation is dependent on RF-DNA (Table 1, lines 5 and 6) and RNA synthesis (Table 1, lines 7 through 9). Preliminary evidence from immunological assays and acrylamide gel electrophoresis suggest that there is preferential synthesis of product proteins from some of the cistrons of the seven known Φ X-174 genomes.^{14–16}

Coupled RNA (RNA derived from the coupled system), uncoupled RNA (RNA synthesized without protein synthesis by eliminating ribosomes and soluble fraction from the complete system), and noncoding RNA (RNA synthesized on the Φ X-174 original strand) were hybridized to Φ X-174 original DNA. Figure 2 shows that there is only hybridization of noncoding RNA which demonstrates strand selectivity during RF-DNA transcription as previously described.¹⁷ When these three RNA's were hybridized to RF-DNA, the saturation plateaus were identical (Fig. 3). Since noncoding RNA represents the whole genome of

FIG. 2.—Hybridization of noncoding RNA (○—○), coupled and uncoupled RNA (●—●) against single-stranded Φ X DNA (0.1 μ g applied to filters).

noncoding RNA represents the whole genome of

FIG. 3.—Hybridization of non-coding, coupled, and uncoupled RNA against RF-DNA (0.2 μ g applied to filters). The saturation plateau of noncoding RNA ($\bigcirc$ — $\bigcirc$) is included in all three figures for comparison. The scale of cpm of noncoding RNA has been adjusted for the difference in base composition.¹⁸ (A) 5 min uncoupled RNA ($\bullet$ — $\bullet$) and 5 min coupled RNA ($\blacksquare$ — $\blacksquare$); (B) 30 min uncoupled RNA ($\bullet$ — $\bullet$) and 30 min coupled RNA ($\blacksquare$ — $\blacksquare$); (C) 30 min uncoupled RNA with ribosomes included in the reaction mixture ($\bullet$ — $\bullet$) or soluble fraction included in the reaction mixture ($\blacksquare$ — $\blacksquare$).

the noncoding strand of RF-DNA, the data of Figure 3 demonstrate that coupled RNA, as well as uncoupled RNA, is the product from the whole genome. This result coincides with a previously published observation that RNA polymerase can transcribe almost the entire genome from RF-DNA *in vitro* (uncoupled).¹⁸ It is in contrast with the *in vitro* transcription of λ ¹⁹ and T₂ or T₄²⁰ where only part of the genome is read. In our coupled system the entire genome is read within the first five minutes of incubation (Fig. 3A). It must be emphasized that the RNA polymerase used in this and previous studies was isolated from uninfected cells of the Φ X-174 host. Since *E. coli* ribosomal and soluble RNA were extracted from the coupled system along with coupled RNA, we demonstrated with a reconstruction experiment that this heterologous RNA had no effect on hybridization under our conditions.

Coupled, uncoupled, and noncoding RNA were not the same size, so it was necessary to demonstrate that size did not affect hybridization. We degraded coupled RNA, the largest of the RNA's, by mild alkaline treatment (pH 12, 0°C, 30 minutes or 6 hours).²¹ Figure 4 shows the RNA profiles in a sucrose gradient; the profile of noncoding RNA, the smallest of the RNA's, is shown in Figure 4A. It is almost identical to that of the six-hour alkaline degraded sample (Fig. 4B).

The hybridization data of Figure 5 illustrate that size had no effect on hybridization within the range of S values measured.

We conclude that with or without concomitant protein synthesis the whole Φ X-174 genome can be transcribed by an *E. coli* RNA polymerase.

FIG. 4.—RNA profiles in a 5–20% sucrose gradient. *E. coli* P³²-RNA (○—○) was added as marker. (A) Noncoding RNA (●---●); (B) 5 min coupled RNA (untreated ●---●) was degraded at pH 12 at 0°C for 30 min (■---■) or 6 hr (▲---▲).

FIG. 5.—Hybridization of alkaline degraded 5 min coupled RNA (see Fig. 4) against RF-DNA (0.03 μ g applied to filters). Untreated (○—○), alkaline-degraded 30 min (□---□) or 6 hr (■---■).

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Abbreviations: RF, replicating form; MSH, mercaptoethanol; PEP, phosphoenolpyruvate; GSH, glutathione; TCA, trichloroacetic acid; PCA, perchloric acid; and SRM, standard reaction mixture.

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