Carbon, J. A., Hung, L., and Jones, D. S. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 979.
Doctor, B. P., Loebel, J. E., Sodd, M. A., and Winter, D. B. (1969), *Science 163*, 693.
Dube, S. K., and Marcker, K. A. (1969), *Eur. J. Biochem. 8*, 256.
Gefter, M. L., and Russell, R. L. (1969), *J. Mol. Biol. 39*, 145.
Harris, C. L., and Titchener, E. B. (1970), 4th Great Lakes Regional Meeting of the American Chemical Society, Fargo, N. D., p 9.
Harris, C. L., Titchener, E. B., and Cline, A. L. (1969), *J. Bacteriol. 100*, 1322.
Hayward, R. S., Eliceiri, G. L., and Weiss, S. B. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 459.
Hayward, R. S., and Weiss, S. B. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1161.
Lipsett, M. N. (1965), *J. Biol. Chem. 240*, 3975.
Lipsett, M. N., Norton, J. S., and Peterkofsky, A. (1967), *Biochemistry 6*, 855.
Lipsett, M. N., and Peterkofsky, A. (1966), *Proc. Nat. Acad. Sci. U. S. 66*, 1169.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Ninio, J., Favre, A., and Yaniv, M. (1969), *Nature (London) 223*, 1333.
Nishimura, S., Yamada, Y., and Ishikuri, H. (1969), *Biochim. Biophys. Acta 179*, 517.
Ohashi, Z., Saneyoshi, M., Harada, F., Hara, H., and Nishimura, S. (1970), *Biochem. Biophys. Res. Commun. 40*, 866.
Peterkofsky, A., and Lipsett, M. N. (1965), *Biochem. Biophys. Res. Commun. 20*, 780.
Reid, B. R. (1968), *Biochem. Biophys. Res. Commun. 33*, 627.
Rushizky, G. W., and Sober, H. A. (1962), *Biochim. Biophys. Acta 55*, 217.
Seno, T., Kobayashi, M., and Nishimura, S. (1969), *Biochim. Biophys. Acta 174*, 71.
von Ehrenstein, G. (1967), *Methods Enzymol. 12*, 588.
Wong, T. W., Weiss, S. B., Eliceiri, G. L., and Bryant, J. (1970), *Biochemistry 9*, 2376.

Template Activities of the ΦX-174 Replicative Allomorphic Deoxyribonucleic Acids*

Yukimasa Hayashi† and Masaki Hayashi‡

ABSTRACT: The template activity of the allomorphs of the replicative form (RF) DNA of ΦX-174 for mRNA synthesis by DNA-dependent RNA polymerase *in vitro* was investigated. When RFI DNA, which does not have a break on either strand, and RFII DNA, derived from RFI by introducing a single-strand break on either strand by DNase, were compared, RFI DNA had a significantly higher rate of transcription. The increased rate was due to the difference of the molecular configuration between these two allomorphs rather than the existence of the nick in RFII. RFI structure increased the stability of the initiation complex between RNA polymerase and the DNA. RNAs synthesized from either RF could be translated to ΦX-174-specific proteins *in vitro*.

The process of transcription of DNA molecules by DNA-dependent RNA polymerase *in vitro* is influenced by at least three factors: environmental conditions such as salt concentration; protein components which may or may not be a constituent of the enzyme; and the molecular structure of the template DNA. For example, the effects of mono- or divalent ions are well documented (Chamberlin and Berg, 1962; Maitra *et al.*, 1967; Richardson, 1969, 1970; Millette and Trotter, 1970). Also, the recent studies of protein factors which have specific roles during the transcription process have led to the discovery of σ (Burgess *et al.*, 1969), ρ (Roberts, 1969), and ψ (Travers *et al.*, 1970). Each has a distinct function during transcription.

The molecular structure of DNA affects the whole transcription scheme as shown in the case of the transcription of denatured DNA (Chamberlin and Berg, 1964a; Sinsheimer and Lawrence, 1964; Bassel *et al.*, 1964). In this case, the RNA product was isolated as an RNA–DNA hybrid until the ratio of template DNA to product RNA reached one. Then free RNA appeared in the reaction mixture. The number of 5′ termini of the product RNA is much greater than the number of RNA polymerase molecules even without adding ρ factor or at low-salt concentration indicating that RNA polymerase can terminate and reinitiate the reaction (Maitra *et al.*, 1967). On the contrary, when native DNA is used as a template under the low-salt condition RNA polymerase stays on the DNA and cannot reinitiate the synthesis of RNA (Bremer and Konrad, 1964). The importance of the configuration or molecular structure of double-stranded DNA for transcription *in vivo* was suggested in the T4 bacteriophage system, in which the DNA must be "competent" to allow

* From the Department of Biology, University of California, San Diego, La Jolla, California 93027. *Received June 21, 1971.* This work was supported, in part, by a U. S. Public Health Service research grant (GM-12934) and by a National Science Foundation research grant (GB-11783). M. H. is the recipient of a Research Career Development award from the U. S. Public Health Service (GM-42360).

† Present address: Institute of Molecular Biology, Faculty of Science, Nagoya University, Nagoya, Japan.

‡ To whom to address correspondence.

late transcription (Riva *et al.*, 1970a,b). In the competent form, DNA is undergoing replication and contains the nicked or gapped regions.

ΦX-174 replicative form (RF)[1] DNA provides another interesting system in which the configuration of double-stranded DNA may affect the transcription process. In *Escherichia coli* infected with ΦX-174, most of the RF assumes either one of two forms (Jansz and Pouwels, 1965; Roth and Hayashi, 1966). The one called RFI sediments in sucrose gradient centrifugation at neutral pH with an *s* value of 21 S. RFI consists of two strands covalently bound into closed double circles. The other form, RFII, has an *s* value of 16 S, and one of the two strands has a nick. RFI can be converted to RFII *in vitro* by pancreatic DNase. As soon as a nick is introduced in either strand of RFI, the whole structure is converted to RFII. Under the electron microscope, RFI assumes a twisted supercoil structure, whereas RFII is observed as an open circle.

RFI and RFII derived by DNase treatment must contain the same base sequence although the hydrodynamic structure is quite different as observed in sucrose gradient centrifugation or electron micrographs. We describe in this paper some of the characteristics of these two allomorphic DNAs as templates of DNA-dependent RNA polymerase.

Methods and Materials

Preparation of ΦX-174 RF DNA is essentially the same as described previously (Hayashi *et al.*, 1963a) except bulk *E. coli* DNA is eliminated prior to the MAK column procedure according to the method of Sugiura *et al.* (1969). *E. coli* C BTCC 122 was grown in SCXD medium (Siegel and Hayashi, 1967) to 5×10^8/ml at 37°. A 4-l. culture was usually processed. Cells were spun down and washed with HF basal medium (Gelfand and Hayashi, 1969) and resuspended in 200 ml of HF basal medium. Wild-type ΦX-174 was added at an moi = 10. The bacteriophage complex was incubated at 37° for 10 min then diluted in 2 l. of prewarmed (37°) SCXD medium (infection time = 0 min). The culture was vigorously aerated through a sparger connected to a sterile compressed air line. At $t = 3$ min, 1 μCi/ml of $[^{32}P]O_4$ was added, and at $t = 8$ min, 50 μg/ml of chloramphenicol was added and incubation was continued to $t = 60$ min. The infected cells were spun down and washed with TES (Tris-HCl (3×10^{-2} M)–EDTA (5×10^{-3} M)–NaCl (0.14 M), pH 8.0) buffer once and resuspended in 84 ml of TES. They were distributed in 12 Spinco polycarbonate tubes (L30 head). Lysozyme (0.4 mg/tube) was then added, and the mixture was incubated at 25° for 15 min. The lysate was diluted to 14 ml with H_2O, and 0.18 ml/tube of 20% SDS was added. After shaking gently at room temperature for 10–20 min, the tubes were spun at 27,000 rpm for 30 min at 10° in a Spinco L30 head. At the end of the run, bulk *E. coli* DNA was precipitated, and the supernatant was carefully collected by a pasteur pipet (~120 ml). One-fifth volumn of 5 M $NaClO_4$, 20 ml of phenol, and 20 ml of chloroform were added and the mixture was shaken at room temperature for 1 hr. After spinning at 5000 rpm for 5 min the water layer was collected. DNA and RNA were precipitated by adding two volumes of ethanol to the water layer. After standing at −20° for 3 hr, the precipitate was collected by centrifugation and resuspended in 80 ml of 0.5 × SSC (1 × SSC is 0.15 M NaCl–0.15 M sodium citrate) and incubated at 37° with 50 μg/ml of pancreatic RNase. The RNase-treated preparation was diluted to 400 ml with 0.5 M NaCl buffered with 0.05 M sodium phosphate at pH 6.5 and chromatogrammed through an MAK column as described previously (Hayashi *et al.*, 1965). Column size was 4× (Hayashi *et al.*, 1965) for 200 ml of the sample. The constant specific activity region ($cpm/OD_{260\ nm}$) was collected and rechromatogrammed through a 2× size (Hayashi *et al.*, 1965) column. Usually at the end of the second column procedure, RF is free from RNase as determined by measuring the digestion of a radioactive RNA preparation at 50° for 24 hr in 0.1 × SSC. The main peak region contains almost exclusively RFI form. The yield of RF was 50–60 $OD_{260\ nm}$ units. Artificial RFII which has only one single-strand nick per molecule was prepared with pancreatic DNase and isolated by sucrose density gradient as described previously (Roth and Hayashi, 1966). Contamination of RFI in RFII is less than 1% as judged by sucrose gradient centrifugation. Heat-denatured RF was made from RFII in 0.1 × SSC by heating at 90° for 10 min followed by quick cooling in ice water.

DNA-dependent RNA polymerase was prepared from frozen *E. coli* C BTCC 122 (ΦX-174 host) as described by Berg *et al.* (1971). Conservation of the structure of allomorphic DNA during incubation with RNA polymerase was tested by comparing the molecular structure of allomorphs before and after RNA synthesis. At the end of the reaction $[^{32}P]PO_4$-labeled RFI or RFII DNA was recovered by phenol extraction. DNA isolated from the RFI DNA primed reaction mixture was examined in a neutral sucrose gradient (Roth and Hayashi, 1966). More than 98% of the ^{32}P counts were recovered as RFI. When RFII DNA was used as the template, DNA isolated at the end of the reaction was sedimented in an alkali gradient (Komano *et al.*, 1968). Two peaks (linear and circular molecules) which had essentially identical radioactive counts were recovered. These experiments indicated that the molecular structure of these allomorphs was conserved during the incubation period.

The in vitro RNA-synthesizing system (uncoupled system) was the transcription part of the transcription–translation-coupled system (coupled system) previously described (Bryan *et al.*, 1969) with a slight modification. The reaction mixture (0.13 ml) contained Tris-HCl (pH 7.9, 1.2×10^{-2} M), $Mg(OAc)_2$ (1.1×10^{-2} M), NH_4Cl (7×10^{-2} M), ATP, GTP, CTP, and $[^3H]$UTP (50 μCi/μmole, 0.385×10^{-3} M each), DNA (15.4 μg/ml), and RNA polymerase (77 μg/ml). Reaction mixtures were incubated at 33°, and at the end of incubation, aliquots were precipitated with ice-cold 6% trichloroacetic acid and trichloroacetic acid insoluble material was collected on a glass-fiber filter (Whatman GF/C) dried and counted in a Beckman L233 scintillation counter.

Coupled system was prepared from *E. coli* Q13 (Bryan *et al.*, 1969). RNA and protein synthesis were determined as in this reference.

Acrylamide gel electrophoresis for ΦX-174-specific proteins (Gelfand and Hayashi, 1969) and RNA (Hayashi and Hayashi, 1970) were described in previous papers. SDS–acrylamide gels were calibrated with respect to the molecular weight and the distance of migration according to Bishop *et al.* (1967). In our gel system, RFII (mol wt 3.4×10^6) migrated with a linear relation between log molecular weight and distance moved; therefore, this marker was used for the calibration of higher molecular weight material.

[1] Abbreviations used are: RF, replicative form; MAK, methylated albumin coated kieselguhr; SDS, sodium dodecyl sulfate; moi, multiplicity of infection.

TABLE I: Asymmetry of RNA Synthesis.[a]

Template for RNA Synthesis	Input, cpm	Hybridization: On Denatured RF (5 μg), cpm	Hybridization: On Single-Stranded DNA (5 μg), cpm	Hybridization: On Denatured T7 DNA (5 μg), cpm
RFI	6590	1969	34	33
RFII	6930	1935	105	33
ΦX-174	5065	1865	2831	46

[a] The condition of RNA synthesis using RFI and RFII was described in Methods and Materials. After 30-min incubation, 25 μg/ml of pancreatic DNase (RNase free) was added. The mixture was incubated for 10 min at 37°. RNA was extracted by phenol method. RNA synthesized using ΦX-174 DNA was prepared according to Bryan *et al.* (1969).

DNA–RNA hybridization was performed in the liquid system described previously (Hayashi and Hayashi, 1968).

Enzymes and Chemicals. Pancreatic DNase (electrophoretically pure, RNase free) was purchased from General Biochemical Co. [^{3}H]UTP was from Schwarz Radiochemicals.

Results

Asymmetric Transcription of RFs. Previously, Hayashi *et al.* (1964) showed that the *in vitro* product from RFI template was complementary to the complementary strand (minus strand) of the original phage strand (plus strand), mimicking *in vivo* strand selection (Hayashi *et al.*, 1963b). Recently, Sugiura *et al.* (1969) showed that active σ component is responsible for asymmetric transcription using

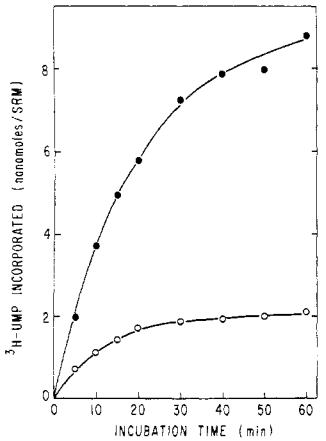

FIGURE 1: Time course of RNA synthesis in uncoupled system. The reaction mixture contained Tris-acetate (pH 7.9, 1.2 × 10^{-2} M), $Mg(OAC)_2$ (1.1 × 10^{-2} M), NH_4Cl (7 × 10^{-2} M), ATP, GTP, CTP, and [^{3}H]UTP (50 mCi/mmole, 3.85 × 10^{-4} M), dithiothreitol (10^{-4} M), and ΦX-174 RF-DNA (15.4 μg/ml). Aliquots were taken at various times after incubation, RNA was precipitated in cold 10% trichloroacetic acid–0.02 M sodium pyrophosphate, and precipitates were collected on glass filters (GF/C Whatman). The filters were washed with cold 6% trichloroacetic acid, dried, and counted in a scintillation counter. RFI-directed RNA synthesis (●). RFII-directed RNA synthesis (○).

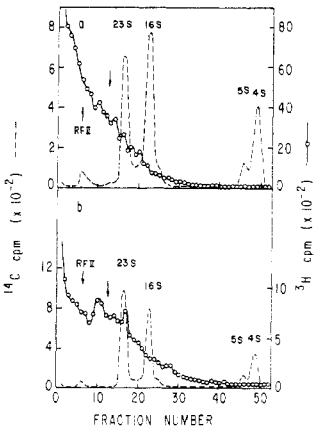

FIGURE 2: Acrylamide gel electrophoresis pattern of RNA synthesized in the uncoupled system. An aliquot from the uncoupl system (Figure 1) after 30-min incubation was diluted with equal volume of sodium acetate (10^{-1} M)–$Mg(OAC)_2$ (pH 5.1, 1C M) and digested with pancreatic DNase (RNase free) at 50 μg/r 0°, for 20 min, Pronase (autodigested to eliminate contaminati RNase) was added to 100 μg/ml, sodium dodecyl sulfate (SDS) 0.2%, and EDTA to 2 × 10^{-3} M. The mixture was passed throu a Sephadex G-50 column (0.9 × 25 cm) to eliminate unincorporat [^{3}H]UTP. RNAs were precipitated with carrier tRNA. (- - -) ^{1} marker total RNA of *E. coli*. (○) [^{3}H]RNA synthesized in the u coupled system (see legend of Figure 1). (a) RFI directed; (RFII directed. The arrow indicates the position of the RN equivalent to the entire genome length (1.7 × 10^6 molecular weigh

fd phage RFI form. Vogt (1969) showed that when co RNA polymerase which does not contain σ factor was use ΦX-174 RFII DNA was transcribed both at double-helic regions and at the nick regions. Table I records the asyı metry of RNA synthesis using RFI and RFII as templat From these data, we concluded that RNA polymerase us in this study contained active σ factor and synthesized RN asymmetrically from either RFI or RFII under the conc tions used in this report. Warnaar *et al.* (1969) show that RFII forms of ΦX-174 which have one to three nicks l DNase on either strand still exercise strand selectivene Our results confirm these observations under different s concentrations.

Kinetics of RNA Synthesis and Size of the Product RN. The time course of RNA synthesis with RFI or RFII templates was examined under the uncoupled conditi (see Methods and Materials, Figure 1). The initial rate RNA synthesis with RFII was about one-third of that wi RFI. The amount of RNA synthesized during the first min from RFI was three- to four-fold more than that fro RFII.

Size distribution of RNA synthesized from RFI and fro RFII was examined by polyacrylamide gel electrophore (Figure 2). We have shown previously (Hayashi *et al.*, 197 that the RNA product from RFI without ρ factor in the rea tion mixture has a molecular weight equal to or higher th the whole genome length indicating RNA polymerase capable of traveling along the template DNA continuous more than one round. Figure 2a also confirms these observ tions. Further, some fractions of the RNA synthesized fro RFII are also larger than the whole genome length. T possibility that the high molecular weight RNA wou result from aggregation of RNA is unlikely since heati RNA in distilled water at 70° for 5 min did not change t pattern. When RNAs synthesized either on RFI or on RF were examined by dimethyl sulfoxide gradient centrifugati

(Strauss *et al.*, 1968), about 40% of the RNA synthesized from RFI sedimented heterogenously between 40 and 30 S. About 10% of the RNA product from RFII was also recovered in this region. RFII DNA with a single nick in one of the two strands sedimented around 30 S. These experiments also indicated that the product RNA from RFI or from RFII contained molecules equal to or larger than the entire genome size.

From the kinetic data (Figure 1) and the size distribution of the product RNA (Figure 2) several conclusions can be drawn. (a) The decreased rate of RNA synthesis on RFII template was not due to the "trapping" of RNA polymerase at the nick (Bautz and Bautz, 1970) resulting in the decrease of the effective concentration of the enzyme since an excess of RNA polymerase over DNA was used in these experiments. (b) When RFII is used as a template, RNA polymerase does not always stop at the nick position which is presumably randomly distributed on either strand of DNA. Otherwise, the product RNA would be equal to or smaller than the whole genome length. The fact that some of the RNA from RFII can assume a molecular weight larger than the whole genome length may be explained by the following argument. If the nick exists on the plus strand, RNA polymerase would continue to move on the minus strand without interference from the nick of the plus strand. On the other hand, if the minus strand has a nick, RNA polymerase would probably be stopped at this point. The decreased count distribution of the higher molecular weight region of Figure 2b (fractions 1–10) and the increased population of RNA species smaller than the whole genome size (fractions 11–50) may indicate the existence of RNA species prematurely terminated by the nick on the minus strand. However, this may not be the only reason for the reduced template activity of RFII in Figure 2 since only half of the RFII molecule would have a nick on the minus strand (see Methods and Materials). If the reduced template activity of RFII is due to the halt of RNA synthesis at the nick on the minus strand, then the rate of RNA synthesis would have been more than one-half of the rate with the RFI template. However, Figure 2 indicates it is about one-third. (c) The reduced template activity of RFII may be due to the difference in molecular structure of RFII and RFI rather than the existence of the nick on RFII. For example, the following possibilities can be visualized. RFI may provide a structure to which more RNA polymerase can bind per RFI molecule than per RFII molecule, or the binding of RNA polymerase to RFI may be more stable. Especially if RNA polymerase can fall off more easily from RFII, this may also explain the reduced population of the higher molecular weight RNA (Figure 2b) and the increased number of RNA species in the smaller molecular weight region.

Recognition of Promoter Sites by RNA Polymerase. The exact mechanism by which RFII exhibits its poor template activity for RNA synthesis is not immediately clear from the preceding experiments. In fact, it may be due to combinations of the possibilities described in part c. However, if the recognition by RNA polymerase of promoter sites is influenced by the difference in molecular structure of the two RF species, it can be tested. Bautz and Bautz (1970) observed that in the presence of σ, *E. coli* RNA polymerase can form a rifampicin-resistant complex with DNA in the absence of nucleoside triphosphates. These complexes only form at promoter sites. Their results also indicated that there is a difference of affinity among T7, T4, λ, and T5 promoters in recognition by *E. coli* polymerase. We carried out a similar experiment using RFI and RFII DNAs. When RFI was used,

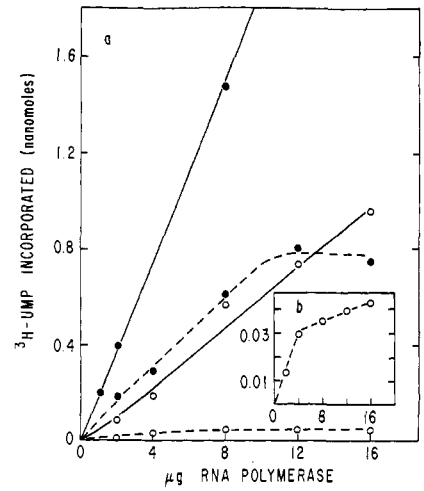

FIGURE 3: Rifampicin-resistant preinitiation complexes of RNA polymerase with ΦX RFI and RFII DNA. Various amounts of RNA polymerase were incubated with 1 μg of RF-DNA in 0.24 ml of Tris-acetate (pH 7.9, 10 μmoles), magnesium acetate (2.5 μmoles), 2-mercaptoethanol (2.5 μmoles), and KCl (12.5 μmoles) at 17°. After 10 min, ATP, GTP, and CTP (50 mμmoles), and [^{3}H]UTP (25 μCi/μmole, 20 mμmoles) in 0.01 ml were added with (broken line) or without (solid line) 1 μg of rifampicin and incubated 5 min at 37°. (A) RFI DNA complex without rifampicin (—●—); RFI DNA complex with rifampicin (- -●- -); RFII DNA complex without rifampicin (—○—); RFII DNA complex with rifampicin (- -○- -). (b) Enlargement of ordinate for RFII DNA complex with rifampicin.

rifampicin-resistant complexes were formed in the system (Figure 3), indicating RNA polymerase recognized the promoter sites and formed a stable complex with RFI. However, the rifampicin-resistant complex between RFII and RNA polymerase was extremely reduced. The apparent ratio of the complex with RFI to that with RFII is about 20 (see the insert of Figure 3).

The RFII structure is presumably the circular structure of linear DNA molecules bound only at the ends of one strand of the DNA, so it should be analogous to the structure of T7 or T4 DNA. Therefore, the recognition mechanism of RNA polymerase of the promoter sites on RFII would be similar to the recognition mechanism of T7 or T4 promoters. In fact, when T7 DNA was used instead of RFII under the same conditions described here, binding of the enzyme to a molecule of DNA was the same order of magnitude (assuming at least one promoter for RFII, and two promoters for T7 according to Bautz and Bautz (1970). Therefore, the apparent increased binding of the enzyme to RFI could result from the fact that the promoter sites on RFI may form a more stable complex with the enzyme. Alternatively, RFI provided 20-fold more promoter sites for RNA polymerase than RFII did. The latter explanation is unlikely because when 5′ termini were measured by the incorporation of β- or γ-labeled nucleotide triphosphates into RNA under the same condition, RFI initiated with three ATPs and two GTPs per molecule. (Hayashi and Hayashi, 1971).

Therefore, the increased amount of rifampicin-resistant complex with RFI suggests an increase of binding affinity of the promoter on RFI for the enzyme. When the ratio of RNA synthesis in the presence and absence of rifampicin is compared, this ratio is much higher with RFI template than RFII (0.68 *vs.* 0.16, Figure 3). This indicates again that RFI forms a more stable complex with the enzyme than RFII does, since

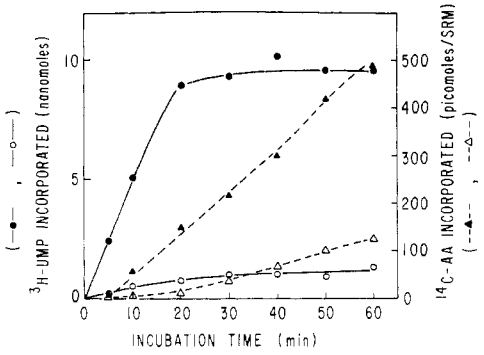

FIGURE 4: Time course of RNA synthesis and protein synthesis in coupled system. Preparation of the coupled system was detailed previously (Bryan *et al.*, 1969). The following modifications were made here: crude initiation factor (20 μg) (Ghosh *et al.*, 1967), leucovorin (10 μg), [^{14}C]amino acids (Arg, Leu, Lys, and Pro) (1.27 mμmoles, (0.29 μCi/mμmoles)), and RNA polymerase (6.6 μg) were added per 130 μl of standard reaction mixture. RNA synthesis dependent on RFI (●); RNA synthesis dependent on RFII (○); protein synthesis RFI dependent RNA directed (▲); protein synthesis RFII dependent RNA directed (△).

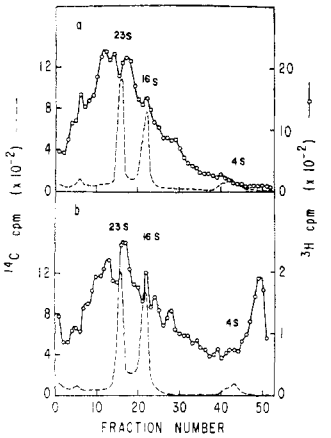

FIGURE 5: Gel electrophoresis pattern of RNA synthesized in the coupled system. RNA samples were prepared from the reaction mixture described in Figure 4. After 30-min incubation aliquots were taken and RNA was purified by phenol method (Bryan *et al.*, 1969). (a) RFI dependent. (b) RFII dependent. (- - -) ^{14}C marker *E. coli* total RNA. (○) [^{3}H]RNA synthesized in the coupled system.

the ratio of RNA synthesis in the presence and absence of rifampicin shows the stability of the complex, not the ratio of the number of promoters.

Messenger Activity of RNAs Synthesized on RFI and RFII. We have previously described the preparation (Bryan *et al.*, 1969) of an *in vitro* DNA-dependent RNA-directed protein-synthesizing system using RF DNA as a template (transcription–translation-coupled system). In this preparation we used RFI DNA exclusively. By comparing ΦX-174 specific *in vivo* protein with *in vitro* product proteins in an SDS–polyacrylamide gel electrophoregram, we concluded that the coupled system can synthesize most of the ΦX-174-specific proteins *in vitro* (Hayashi *et al.*, 1970). In particular, one of the *in vivo* and *in vitro* proteins (G protein) was subjected to tryptic digestion and the digested peptides were fingerprinted. The results indicated that coupled system faithfully translated the mRNA to final protein products (Gelfand and Hayashi, 1970). The coupled system provides an excellent opportunity to examine translatability of an RNA product derived from RFII. Figure 4 shows the kinetics of RNA and protein synthesis programmed by RFI and RFII. RFII has poor template activity for RNA synthesis in the coupled system. However, the synthesized RNA from RFII was translated with almost the same efficiency as evidenced by the ratio of amino acids incorporated *vs.* UMP incorporated (Figure 4). Furthermore, the molecular weight of RNA 30 min after incubation was compared. The molecular weight distribution of the RNA synthesized from RFI or RFII was almost identical around the high molecular weight region (Figure 5). The appearance of the low molecular weight RNA in the RFII template preparation is not fully understood. In general, the RNA in the coupled system tends to be smaller than the RNA from uncoupled synthesis (compare Figure 2 and Figure 5). This may indicate the existence of RNase activity or specific terminators (*e.g.*, ρ) in the preparation. Finally the product proteins were analyzed by an SDS–acrylamide gel electrophoresis. Figure 6 shows the results of this type of experiment. When RFI was used as a template of the system, we synthesized X, D, G, B/I, and F proteins and probably A and H proteins (Figure 6Z). Peak Z is frequently found in the *in vitro* product and in some *in vivo* preparations (Hayashi *et al.*, 1970). A very similar pattern of the product proteins from RFII was also observed (Figure 6b). This indicates to us that RFII could synthesize translatable RNA species under the coupled condition.

Discussion

The results described in the preceding section indicate that the difference of the template activities between RFI and RFII can be attributed, at least in part, to the difference of the molecular structure of these two allomorphs rather than the existence of the nick on RFII. The increased competency of RFI over RFII as a template for transcription is possibly due to the increased stability of the initiation complex as shown by the rifampicin experiments (Figure 3).

The twisted form of RFI results from deficient turns in the Watson–Crick helix as shown by Vinograd and Lebowitz (1966) and Crawford and Waring (1967) in the case of polyoma DNA. A recent study by Dean and Lebowitz (1971) showed that superhelical DNAs contain unpaired bases and that supercoiling causes alterations in the double-helical structure of native, closed, circular DNA. This observation indicates to us that these unpaired bases (single-stranded region) would provide a greater binding affinity for RNA polymerase. Wood and Berg (1964) and Chamberlin and Berg (1964b) suggested that all RNA polymerase binding sites on double-helical DNA may be single-stranded regions. Richardson (1966) showed by competition studies that on the average the affinity of RNA polymerase for sites on f1 DNA may be as much as three times greater than on T7 DNA. Richardson (1966) also found by direct binding studies that there are more sites per unit weight on single-stranded DNA than on a double-helical DNA. The smaller number of sites on normally double-helical DNAs would be those rare places of single strandedness, such as at points where there is a collapse of the normally helical structure.

Dean and Lebowitz's (1971) observation of the existence of unpaired bases on the supercoiled DNA does not include the number and size of such unpaired base sequences per molecule. If our speculation that such unpaired bases would provide stronger binding sites for RNA polymerase is correct, the unpaired bases would preferentially exist at the promoter sites. Since the structure of DNA is dynamic (von Hippel

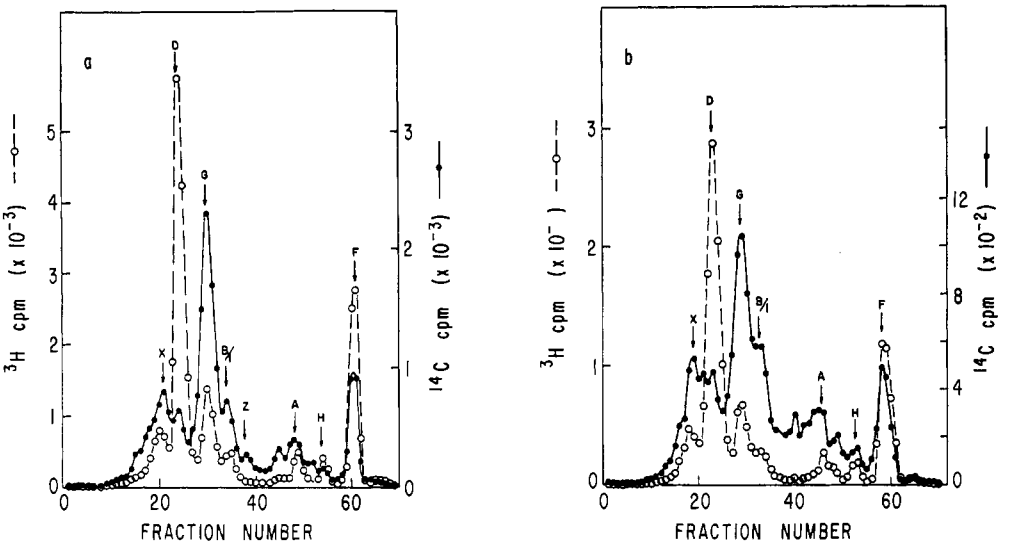

FIGURE 6: Gel electrophoresis pattern of protein synthesized in the coupled system. (a) RFI dependent RNA directed. (b) RFII dependent RNA directed. (●) ^{14}C marker *in vivo* protein synthesized in uv-irradiated and wild-type ΦX-174 infected cells (Gelfand and Hayashi, 1969); (○) [^{3}H]protein synthesized in coupled system (30 min). The new designation of the cistrons which was agreed upon at the small DNA phage conference held at the California Institute of Technology on Nov 7, 1970, is adapted in this figure. In order to avoid confusion, the new designation and old desigantions are listed below.

New	ΦX-174 (Sinsheimer)	ΦX-174 (Hayashi)	S-13 (Tessman)
A	VI	C	IV
B	IV	B	II
C	VIII	H	VI
D	V	D	VII
E	I	G	V
F	VII	E	I
G	III	F	III_a
H	II	A	III_b
I		I	

X is a phage structural protein, cistron X has not been identified.

and Printz, 1964), there might be sufficient unwinding and rewinding of the helix at any given locus. However, because of the postulated specific base sequences of the promoter sites (Szybalski *et al.*, 1966), it would be possible that such unpaired bases would accumulate at the promoter sites.

We have tried a preliminary experiment which compared the difference of the template activity for RNA synthesis *in vitro* between the linear form of λ DNA and the intracellular supercoiled circular DNA of λ (Young and Sinsheimer, 1964). In this case, the intracellular supercoiled DNA showed a threefold better template activity than the linear molecules in terms of amount of RNA synthesized. This difference, at least in part, was attributed to the difference in the affinity of RNA polymerase for the DNA.

Hayashi (1965) and Hayashi and Hayashi (1966, 1968) observed that during *in vitro* and *in vivo* transcription processes of ΦX-174 a small segment of the newly synthesized RNA existed as DNA–RNA hybrid complex which is phenol and duponol treatment resistant and pancreatic RNase resistant. The complex was dissociated to RF DNA and RNA after heating in low salt or by formamide treatment. As the process proceeded, the RNase-resistant region became RNase sensitive, so it was concluded that the most recently synthesized RNA is temporarily hybridized to RF DNA. Contrary to these observations, when linear DNA is used as a template, the temporal DNA–RNA hybrid complex could not be detected (Bremer and Konrad, 1964; see also Richardson's review, 1969). Presumably, in the case of transcription of ΦX-174 RFI DNA, RNA polymerase recognized the unpaired bases at the promoter. As the transcription process proceeded, the unpaired promoter sites may be base paired and the unpaired region moved together with RNA polymerase along the circular structure, thus stabilizing the newly synthesized portion of the RNA as the temporal DNA–RNA hybrid.

In the present study, we used RFII derived *in vitro* from RFI by DNase nicking. However, the identical results have been obtained when "naturally occurring" RFII (nRFII) was used. The nRFII form was purified from the tailing part of the MAK column. As previously shown (Roth and Hayashi, 1966), RFII can be separated from RFI by MAK chromatography. The template activity of nRFII is of the same order as that of RFII derived from RFI with DNase. However, we have no clear evidence that our nRFII preparation is not the product of the conversion from RFI during the isolation process. Therefore, our results concerning nRFII have some reservation.

It is evident from the experiments by Knippers *et al.* (1968), Komano *et al.* (1968), and Schekman *et al.* (1971) that there exists naturally occurring RFII with a nick or a gap on the specific strand. Further, Komano *et al.* (1968), Knippers *et al.* (1968), and Gilbert and Dressler (1968) showed that the RFII is the template for the single-stranded DNA. It is an interesting question to ask which form of RF can serve as the template for the ΦX-174 message *in vivo*. Especially, since the asymmetric synthesis of message *in vitro* is possible on both RFs, and in the *in vitro* protein-synthesizing-coupled system both RFs can be the template for ΦX-174-specific protein messages, there must be some selective mech-

anism *in vivo* to choose the correct template if only one of the two forms serves as the *in vivo* template.

Acknowledgment

We thank D. H. Gelfand for the gift of T7 DNA.

References

Bassel, A., Hayashi, M., and Spiegelman, S. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 796.

Bautz, E. K. F., and Bautz, L. A. (1970), *Nature (London) 226*, 1219.

Berg, D, Barrett, K., and Chamberlin, M. (1971), *Methods Enzymol. 21*, 506.

Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S. (1967), *J. Mol. Biol. 26*, 373.

Bremer, H., and Konrad, M. W. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 801.

Bryan, R. N., Sugiura, M., and Hayashi, M. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 483.

Burgess, R. R., Travers, A. A., Dunn, J. J., and Bautz, E. K. F. (1969), *Nature (London) 221*, 43.

Chamberlin, M., and Berg, P. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 81.

Chamberlin, M., and Berg, P. (1964a), *J. Mol. Biol. 8*, 289.

Chamberlin, M., and Berg, P. (1964b), *J. Mol. Biol. 8*, 708.

Crawford, L. V., and Waring, M. J. (1967), *J. Mol. Biol. 25*, 23.

Dean, W. W., and Lebowitz, J. (1971), *Nature (London) 231*, 5.

Gelfand, D. H., and Hayashi, M. (1969), *J. Mol. Biol. 44*, 501.

Gelfand, D. H., and Hayashi, M. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 13.

Ghosh, H. P., Soll, D., and Khorana, H. G. (1967), *J. Mol. Biol. 25*, 275.

Gilbert, W., and Dressler, D. (1968), *Cold Spring Harbor Symp. Quant. Biol. 33*, 473.

Hayashi, M. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 1736.

Hayashi, M., Hayashi, M. N., and Spiegelman, S. (1963a), *Science 140*, 1313.

Hayashi, M., Hayashi, M. N., and Spiegelman, S. (1963b), *Proc. Nat. Acad. Sci. U. S. 50*, 664.

Hayashi, M., Hayashi, M. N., and Spiegelman, S. (1964), *Proc. Nat. Acad. Sci U. S. 51*, 351.

Hayashi, M. N., and Hayashi, M. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 635.

Hayashi, M. N., and Hayashi, M. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1107.

Hayashi, M. N., and Hayashi, M. (1971), *J.* Virol. (in press).

Hayashi, M. N., Hayashi, M., and Hayashi, Y. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 174.

Hayashi, M. N., Hayashi, M., and Spiegelman, S. (1965), *Biophys. J. 5*, 231.

Hayashi, Y., and Hayashi, M. (1970), *Cold Spring Harbor Symp. Quant. Biol. 35*, 171.

Jansz, H. S., and Pouwels, P. H. (1965), *Biochem. Biophys. Res. Commun. 18*, 589.

Knippers, R., Komano, T., and Sinsheimer, R. L. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 577.

Komano, T., Knippers, R., and Sinsheimer, R. L. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 911.

Maitra, V., Nakata, Y., and Hurwitz, J. (1967), *J. Biol. Chem. 242*, 4908.

Millette, R. L., and Trotter, C. D. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 701.

Richardson, J. P. (1966), *J. Mol. Biol. 21*, 83.

Richardson, J. P. (1969), *Progr. Nucl. Acid Res. Mol. Biol. 9*, 75.

Richardson, J. P. (1970), *Nature (London) 225*, 1109.

Riva, S., Cascino, A., and Geiduschek, E. P. (1970a), *J. Mol. Biol. 54*, 85.

Riva, S., Cascino, A., and Geiduschek, E. P. (1970b), *J. Mol. Biol. 54*, 103.

Roberts, J. W. (1969), *Nature (London) 224*, 1168.

Roth, T. F., and Hayashi, M. (1966), *Science 154*, 658.

Schekman, R. W., Iwaya, M., Bromstrup, K., and Denhardt, D. T. (1971), *J. Mol. Biol. 57*, 177.

Siegel, J., and Hayashi, M. (1967), *J. Mol. Biol. 27*, 443.

Sinsheimer, R. L., and Lawrence, M. (1964), *J. Mol. Biol. 8*, 289.

Strauss, J. H., Jr., Kelly, R. B., and Sinsheimer, R. L. (1968), *Biopolymers 6*, 793.

Sugiura, M., Okamoto, T., and Takanami, M. (1969), *J. Mol. Biol. 36*, 125.

Szybalski, W., Kubinski, H., and Sheldrick, P. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 123.

Travers, A. A., Kamen, R. I., and Schleif, R. F. (1970), *Nature (London) 228*, 748.

Vinograd, J., and Lebowitz, J. (1966), *J. Gen. Physiol. 49*, 103.

Vogt, V. (1969), *Nature (London) 223*, 854.

von Hippel, P. H., and Printz, M. P. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 1458

Warnaar, S. O., Mulder, G., Van der Sigtenhorst-Van der Sluis, I., Van Kesteren, L. W., and Cohen, J. A. (1969), *Biochim. Biophys. Acta 174*, 239.

Wood, W. B., and Berg, P. (1964), *J. Mol. Biol. 9*, 452.

Young, E. T., II, and Sinsheimer, R. L. (1964), *J. Mol. Biol. 10*, 562.