Hough, B. R., and Davidson, E. H. (1972), *J. Mol. Biol. 70,* 491.
Hoyer, B. H., Rice, N. R., and Van de Velde, N. W. (1973), *Carnegie Inst. Wash. Yearb. 73,* 214.
Irving, C. C., (1973), *Methods Cancer Res. 7,* 189.
Kelly, R. B., Atkinson, M. R., Huberman, J. A., and Kornberg, A. (1969), *Nature (London) 244,* 495.
Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I., and Khorana, H. G. (1971), *J. Mol. Biol. 56,* 341.
Kushner, S. R., Kaplan, J. C., Ono, H., and Grossman, L. (1971), *Biochemistry 10,* 3325.
Laird, C. D., McConaughy, B. L., and McCarthy, B. J. (1969), *Nature (London) 224,* 149.
Legator, M. S., and Flamm, W. G. (1973), *Annu. Rev. Biochem. 42,* 683.
Lehmann, A. R. (1972), *J. Mol. Biol. 66,* 319.
Lieberman, M. W. (1973), *Biochim. Biophys. Acta 324,* 309.
Lieberman, M. W., and Forbes, P. D. (1973), *Nature (London), New Biol. 241,* 199.
Lieberman, M. W., and Poirier, M. C. (1973), *Cancer Res. 33,* 2097.
Lieberman, M. W., and Poirier, M. C. (1974a), *Proc. Nat. Acad. Sci. U. S. 71,* 2461.
Lieberman, M. W., and Poirier, M. C. (1974b), *Biochemistry 13,* 3018.
Lieberman, M. W., Rutman, J. Z., and Farber, E. (1971), *Biochim. Biophys. Acta 247,* 497.
Loeb, L. A., Tartof, K. D., and Travaglini, E. C. (1973), *Nature (London) New Biol. 242,* 66.
McConaughy, B. L., and McCarthy, B. J. (1970), *Biochem. Genet. 4,* 425.
Miller, J. A. (1970), *Cancer Res. 30,* 559.
Regan, J. D., Setlow, R. B., and Ley, R. D. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 708.
Setlow, R. B., and Regan, J. D. (1972), *Biochem. Biophys. Res. Commun. 46,* 1019.
Setlow, R. B., Regan, J. D., and Carrier, W. L. (1972), *Biophys. Soc. Abs. 12,* 19a.
Sneider, T. W. (1971), *J. Biol. Chem. 246,* 4774.
Springgate, C. F., and Loeb, L. A. (1973), *Proc Nat. Acad. Sci. U. S. 70,* 245.
Stich, J. F., and Laishes, B. A. (1973), *Pathobiology Annu. 3,* 341.
Sutton, W. D. (1971), *Biochim. Biophys. Acta 240,* 522.
Ullman, J. S., and McCarthy, B. J. (1973), *Biochim. Biophys. Acta 294,* 416.
Vogt, V. M. (1973), *Eur. J. Biochem. 33,* 192.

Isolation of DNA Segments Containing Promoters from Bacteriophage T3 DNA†

T. Takeya and M. Takanami*

ABSTRACT: In order to isolate DNA segments containing promoters, bacteriophage T3 DNA was cleaved into about 60 unique fragments with endonuclease · Hap, which introduces duplex cleavages at the sequence of

---C/C-G-G---
---G-G-C/C---

(Sugisaki, H., and Takanami, M. (1973), *Nature (London), New Biol. 246,* 138). The specificity of RNA initiation was not influenced by this digestion, as far as the number of initiation and starting sequences for RNA synthesis was concerned. When the digest was incubated with either *Escherichia coli* RNA polymerase holoenzyme or T3 RNA polymerase, each enzyme was shown to form stable complexes with specific fragments depending on the combination of nucleoside triphosphates present in the binding mixture. The selectivity of binding by the host polymerase was lost if the σ factor was removed from the enzyme. Evidence that promoters are contained in the fragments isolated by the specific interaction with the polymerases was demonstrated with the fragment derived from the left end of T3 DNA. The procedures used in the present study should make possible isolation of DNA segments containing promoters from any DNA molecule.

Since the role of σ factor in the selection of initiation sites by RNA polymerase was elucidated (Burgess *et al.*, 1969; Krakow *et al.*, 1969; Travers and Burgess, 1969), a number of investigations have focused on the molecular mechanism of RNA initiation. However, little information is as yet available concerning the structure of RNA at which RNA synthesis is initiated. One of the difficulties in isolating the RNA initiation site (promoter) is that RNA polymerase can tightly bind to single-stranded regions of DNA, which are often created by the breakage of DNA (Hinkle *et al.*, 1972; Okamoto *et al.*, 1972). Recently, restriction endonucleases which degrade foreign DNA at a limited number of sites have been isolated from a variety of bacterial strains (for reviews, see: Boyer, 1971; Meselson *et al.*, 1972). As this type of enzyme introduces duplex cleavages on double-stranded DNA at a specific sequence of several nucleotides (Kelly and Smith, 1970, Hedgpeth *et al.*, 1972; Bigger *et al.*, 1973; Boyer *et al.*, 1973), these cleavages may not create nonspecific binding sites for RNA polymerase at the terminal regions. Accordingly, experiments were designed to isolate DNA segments containing promoters from bacteriophage T3 DNA after the cleavages with this type of enzyme. T3 DNA is known to provide at least two types of

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promoters which are respectively recognized by host and phage-specific RNA polymerases (Maitra, 1971; Dunn *et al.*, 1971). It is therefore easy to compare the specificity of the isolated fragments with these polymerases. As the restriction endonucleases, three species of enzymes isolated from *Haemophilus* strains were used in the present study (Takanami, 1973). These enzymes are abbreviated as Hap, Hga, and HinH according to the nomenclature proposed by Smith and Nathans (1973).[1] When T3 DNA was treated with Hap, which cleaves DNA at a sequence of

---C/C-G-G---
---G-G-C/C---

(Sugisaki and Takanami, 1973), this DNA molecule was cleaved into about 60 unique fragments. As the specificity of RNA initiation was not influenced by this digestion, DNA fragments containing promoters were successfully isolated from the digest by the specific interaction with RNA polymerase.

Materials and Methods

T3 DNA. Phage T3 DNA was replicated in *Escherichia coli* R11, collected, and purified by CsCl density-gradient centrifugation. DNA was extracted by a mild phenol treatment and dialysis (Takeya and Fujisawa, 1973). For the preparation of T3 DNA uniformly labeled with ^{32}P (about 10^7 cpm/A_{260} unit), the phage was replicated in the host cells, growing in a Tris-glucose medium containing ^{32}P (1 mCi/100 ml).

Host and T3 RNA Polymerases. E. coli RNA polymerase was prepared from *E. coli* A19 (Takanami *et al.*, 1971), and converted to the holoenzyme by adding an excess amount of the σ fraction which was prepared by the method of Burgess *et al.*, (1969). The core enzyme was prepared from the polymerase fraction by two cycles of phosphocellulose column chromatography (Burgess *et al.*, 1969). T3 RNA polymerase was purified from T3-infected *E. coli* R11 by applying the method of Chamberlin *et al.* (1970). The purified T3 polymerase strictly required T3 DNA as template and was insensitive to rifampicin (Maitra, 1971; Dunn *et al.*, 1971).

Restriction Endonucleases. Hap, Hga, and HinH were purified from three *Haemophilus* strains: *H. aphirophilus, H. gallinarum,* and *H. influenzae* H-I, as described previously (Takanami, 1973).

Digestion of DNA by Restriction Endonucleases. About 200 units of enzyme was added per 1 A_{260} unit of DNA, and the mixture was incubated for 6 hr at 37° in 10 mM Tris (pH 7.6)–7 mM $MgCl_2$–7 mM mercaptoethanol. The reaction was terminated by shaking with 80% phenol. The separated aqueous layer was treated with ethyl ether, briefly dialyzed against 20 mM Tris (pH 7.6)–0.1 mM EDTA, and used for the experiment.

Polyacrylamide Gel Electrophoresis of DNA Fragments. The digest of DNA was layered on 3–10% gel columns (0.6 cm × 12 cm) formed in 0.036 M Tris–0.032 M KH_2PO_4–0.1 mM EDTA (pH 7.8), and electrophoresed for 16 hr at 2 mA/tube. For the autoradiography, gels were longitudinally split into halves, covered with thin plastic films, and exposed to X-ray films.

Nitrocellulose Filter Assay for the Binding of RNA Polymerase to DNA. The binding of RNA polymerase to DNA was assayed by the nitrocellulose filter method (Hinkle and Chamberlin, 1972). Unless otherwise noted, the binding mixture contained 2 μg (about 10^6 cpm) of [^{32}P]DNA, 10 μg of host polymerase or 2 μg of T3 polymerase, 0.4 mM each of indicated nucleoside triphosphates (NTP), 8 mM $MgCl_2$, 50 mM Tris (pH 7.8), and 150 mM KCl (for host polymerase) or 30 mM KCl (for T3 polymerase), in a final volume of 1 ml. Following incubation for 5 min at either 27 (for host polymerase) or 37° (for T3 polymerase), 10 μg of an Hap digest of nonlabeled T3 DNA was added. After 3 min, the mixture was passed through a nitrocellulose filter (Millipore HA 0.45 μ). The filter was washed with 30 ml of the washing buffer (50 mM Tris (pH 7.8)–8 mM $MgCl_2$) containing 1 M KCl (for host polymerase) or 0.2 M KCl (for T3 polymerase), with a flow rate of 2 ml/min. ^{32}P retained on the filter was determined. To recover DNA fragments, the washed filter was incubated for 1 hr at 37° in 2 ml of 0.1% sodium dodecyl sulfate, and the eluate was shaken with 80% phenol. The separated aqueous layer was treated with ethyl ether and dialyzed against 20 mM Tris (pH 7.6)–0.1 mM EDTA.

Synthesis and Size Determination of RNA. RNA synthesis was carried out in the reaction mixture (1 ml) containing 8 mM $MgCl_2$, 50 mM Tris (pH 7.8), 0.1 mM dithiothreitol, 0.4 mM [^3H]ATP (6000 cpm/nmol), 0.4 mM each of three other nucleoside triphosphates, 10 μg of DNA, 10 μg of host polymerase or 2 μg of T3 polymerase, and 150 mM KCl (for host polymerase) or 30 mM (for T3 polymerase). When the starting termini of RNA were labeled, ATP or GTP was replaced by [γ-^{32}P]ATP or [γ-^{32}P]GTP (about 10^7 cpm/nmol), respectively. Incubation for 20 min at 37° was terminated by shaking with 80% phenol. The separated aqueous layer was passed through a Sephadex G100 column (1 cm × 30 cm), equilibrated with 0.14 M NaCl–0.02 M Tris (pH 7.6). The RNA-containing fraction was then centrifuged on a sucrose density gradient (5–20% in 0.14 M NaCl–0.02 M Tris (pH 7.6).

Analysis of the Starting Nucleotide Sequences of RNA. RNA labeled with either [γ-^{32}P]ATP or [γ-^{32}P]GTP was prepared as in the above section, and hydrolyzed with pancreatic RNase. The resulting oligonucleotides were chromatographed on DEAE-Sephadex A25 columns, using the conditions previously described (Sugiura *et al.*, 1969). Dinucleotides with different sequences were resolved by Dowex I column chromatography.

Terminal Labeling of DNA. Labeling of the 5′ termini of DNA in the polynucleotide kinase reaction was carried out according to Richardson (1966).

Results

Initiation of RNA Synthesis on the Hap Digest of T3 DNA. When T3 DNA was exhaustively digested with Hap and fractionated be gel electrophoresis, the pattern of digestion products seen in Figure 1 was obtained. The pattern was very reproducible and was not altered by further incubation. The number of fragments produced was estimated to be about 60. The fragments were numbered as Hap 1, Hap 2, etc. in order of decreasing size. To identify the terminal DNA fragments, nonlabeled T3 DNA was treated with alkaline phosphatase and rephosphorylated with ^{32}P in the polynucleotide kinase reaction. Following digestion with Hap, ^{32}P was found at two discrete regions, corresponding to Hap 1 and Hap 10 or 11 (Figure 2A).

The complete Hap digest of T3 DNA was added to the

[1] Abbreviations used are: Hap, Hga, and HinH, enzyme species from *Haemophilus aphirophilus, H. gallinarum,* and *H. influenzae* H-I, respectively; NTP, nucleoside triphosphate.

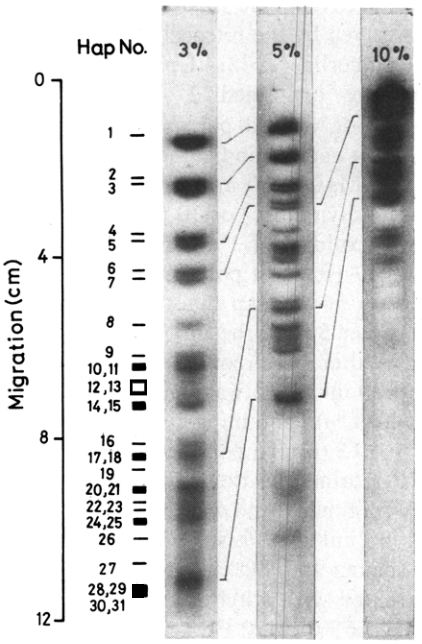

FIGURE 1: Gel electrophoresis of the Hap digest of T3 DNA. The Hap digest of ^{32}P-labeled T3 DNA was layered on 3, 5, and 10% gel columns and electrophoresed for 16 hr at 2 mA/tube. The band numbers of the resolved fragments are given by the side of the autoradiograph.

RNA synthesizing system containing host RNA polymerase and either [γ-^{32}P]ATP or [γ-^{32}P]GTP, and the number of RNA chains formed was compared with that on intact T3 DNA. As shown in Table I, neither incorporation of [α-^{32}P]ATP nor [γ-^{32}P]GTP into RNA was influenced by the digestion with Hap. When the synthesized RNA was hydrolyzed with pancreatic RNase and the resulting terminal oligonucleotides were analyzed, the same results were obtained as with RNA made on intact T3 DNA. About 85% of the (pppA- - -)RNA was initiated with pppAU- - - and the remainder with pppAC- - -, while the (pppG- - -)RNA was mostly initiated with pppGU- - -.

Nucleoside Triphosphate Dependent Binding of DNA Fragments of RNA Polymerases. Major RNAs formed on T3 DNA by the host polymerase are initiated with pppAUG- - - and pppGU- - - and those by the T3 polymerase with pppG(Pu)$_n$Py- - - (n = 2, 4, 5, and 6) (Takeya and Fujisawa, 1973). As it has been suggested that the formation of the first phosphodiester bond by the initiating nucleoside triphosphates greatly stabilizes the DNA–RNA polymerase complex (Anthony *et al.*, 1969), the Hap digest of [^{32}P-labeled] T3 DNA was mixed with either the host polymerase or T3 polymerase in the presence or absence of a mixture of ATP, GTP, and UTP. Following a brief incubation, the mixtures were passed through nitrocellulose filters which bind these polymerases. The filters were washed with different concentrations of KCl, and the [^{32}P]DNA retained on the filters as the polymerase complex was determined. In the absence of NTP's, ^{32}P retained on filters at low salt was dissociated by washing the filters with high salt. In the presence of NTP's, however, a significant amount of ^{32}P was retained even after washing with high salt (Figure 3). Other combinations of three NTP's enabled the host polymerase to bind considerable amounts of ^{32}P at high salt. However, the addition of one or two species of NTP's did not markedly stabilize the complex with the host polymerase, except for the combination of ATP + CTP (Table II). These results support previous claims that the

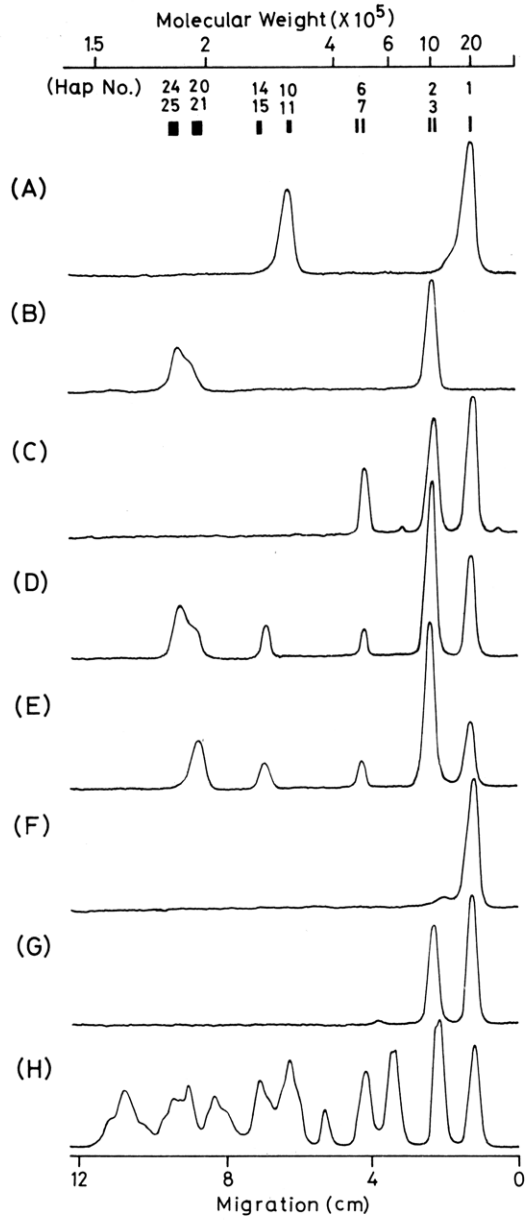

FIGURE 2: Analysis of the fragments derived from the termini (A) and those isolated by the interaction with T3 and host RNA polymerases (B–H). (A) Intact T3 DNA was treated with alkaline phosphatase and rephosphorylated with ^{32}P in the polynucleotide kinase reaction. The labeled DNA was digested with Hap, layered on a 3% gel column, and electrophoresed for 16 hr at 2 mA/tube. The resulting autoradiograph was traced by a densitometer. (B–H) The Hap digest of ^{32}P-labeled T3 DNA was incubated with either host polymerase or T3 polymerase in the presence of NTP's in different combinations. The DNA fragments which formed complexes with the polymerases were isolated as in the legend to Table III. The fragments were resolved on gel electrophoresis, and the autoradiographs were traced as above. The correlation between migration distance *vs.* molecular weight was estimated from the relative mobilities to the fragments produced from fd RF-I DNA by the cleavage with Hap (Takanami, 1973): (B) host polymerase + ATP, CTP, and UTP; (C) host polymerase + ATP, GTP, and UTP; (D) host polymerase + ATP, CTP, and GTP; (E) host polymerase + CTP, GTP, and UTP; (F) T3 polymerase + ATP; (G) T3 polymerase + ATP, GTP; (H) host polymerase core enzyme + ATP, CTP, GTP.

binding of RNA polymerase to DNA is stabilized by the formation of oligonucleotides at the RNA initiation sites.

In contrast to the DNA binding of host RNA polymerase, the binding of T3 RNA polymerase to DNA was stabilized with GTP alone (Table II). As the T3 polymerase has recently been shown to initiate RNA with pppGG- - - (Chakraborty *et al.*, 1973), it appears that the formation of G-

TABLE I: Effect of Hap Digestion on the Formation of RNA Termini.[a]

Template	Incorporation (pmol) [^{32}P]ATP	[^{32}P]GTP
Intact T3 DNA	0.56	0.38
Hap digest	0.66	0.40

[a] Intact T3 DNA or the Hap digest of T3 DNA was incubated for 10 min at 37° in RNA synthesizing mixtures containing host RNA polymerase and either [γ-^{32}P]ATP or [γ-^{32}P]GTP. Synthesized products were isolated, and ^{32}P incorporated into RNA was determined. The composition of the reaction mixtures and procedure for the isolation of synthesized products are given in the Materials and Methods section.

TABLE II: Effect of Nucleoside Triphosphates on the Formation of Stable DNA–RNA Polymerase Complexes.[a]

Nucleoside Triphosphates	^{32}P Retained (cpm, over Background) Host RNA Polymerase	T3 RNA Polymerase
A	0	0
U	0	0
G	24	*680*
C	0	0
A + U	120	0
A + C	*817*	0
A + G	168	*2920*
G + C	184	*802*
G + U	0	*1043*
A + G + U	*755*	
A + C + G	*1955*	
A + C + U	*669*	
G + C + U	*1025*	

[a] The Hap digest of ^{32}P-labeled T3 DNA (0.4 μg, 5 $\times$ 10^4 cpm) was incubated as indicated under Materials and Methods in the binding mixture (1 ml) containing either host polymerase (2 μg) or T3 polymerase (0.4 μg), in the presence of indicated species of NTP. Following addition of a Hap digest of nonlabeled T3 DNA (2 μg), the mixtures were passed through nitrocellulose filters. The filters were washed with wash buffer containing 1 M KCl (for host polymerase) or 0.2 M KCl (for T3 polymerase), and the amount of ^{32}P retained was determined. Without adding NTP to the binding mixtures, ^{32}P retained on a filter was an average 425 cpm with host polymerase and 190 cpm with T3 polymerase. These values were subtracted as background. Values showing significant binding are in italics.

TABLE III: Reassociation Ability of DNA Fragments Dissociated from the Complexes with RNA Polymerase.[a]

Comp. of NTP's 2nd Incubation	^{32}P Retained (cpm, over Background)[b] Fragments from Complexes Formed with ATP + CTP (11,000 cpm/Tube)	Fragments from Complexes Formed with ATP + GTP + UTP (5600 cpm/Tube)
A + U	0	0
A + G	249 (2.3)	719 (12.8)
A + C	*2965* (26.7)	0
G + U	0	361 (6.4)
G + C	239 (2.1)	478 (8.5)
A + G + U	441 (4.0)	*1641* (29.3)
A + C + G	*5193* (47.1)	*2198* (39.3)
A + C + U	*2953* (26.9)	0
G + C + U	312 (2.8)	*1001* (17.9)

[a] The Hap digest of ^{32}P-labeled T3 DNA was incubated in the binding mixtures (1 ml each) containing host RNA polymerase and either ATP + CTP or ATP + GTP + UTP, and the DNA–RNA polymerase complexes formed were retained on nitrocellulose filters, as described under Materials and Methods. The complexes were eluted from the filters by incubating for 1 hr at 37° in 0.2% sodium dodecyl sulfate. The eluates were shaken with 80% phenol. The aqueous layers were treated with ethyl ether and dialyzed against 20 mM Tris (pH 7.6)–0.1 mM EDTA. [^{32}P]DNA fragments thus prepared were added to the binding mixtures containing host polymerase and NTP's in the indicated combinations. The mixtures were incubated and passed through nitrocellulose filters, and ^{32}P retained was determined. Values showing significant binding are in italics. [b] Values in parentheses are per cents.

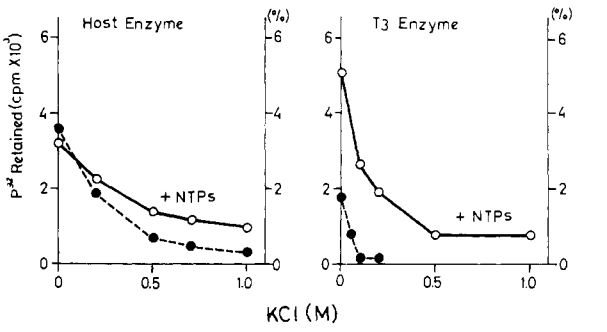

FIGURE 3: Stability of the DNA–RNA polymerase complexes. The Hap digest of ^{32}P-labeled T3 DNA (0.4 μg, 5 $\times$ 10^4 cpm) was incubated in the binding mixture (1 ml) containing either host polymerase (2 μg) or T3 polymerase (0.4 μg), in the presence or absence of a NTP mixture (ATP, GTP,and UTP, 0.4 mM each). An Hap digest of nonlabeled T3 DNA (2 μg) was added. The mixtures were passed through nitrocellulose filters. The filters were washed with wash buffer containing indicated concentrations of KCl. ^{32}P retained on the filter was determined.

oligonucleotides is sufficient to stabilize initiation complexes.

DNA fragments retained on nitrocellulose filters by forming stable complexes with the host polymerase in the presence of either ATP + CTP or ATP + GTP + UTP were eluted from the filters (abbreviated as AC fragments and AGU fragments), and their reassociation ability toward the host polymerase was examined. As shown in Table III, the AC fragments were efficiently rebound by the polymerase with NTP mixtures containing ATP and CTP, while the AGU fragments were efficiently rebound with the combination of ATP + GTP + UTP, as expected. However, the AGU fragments also bound to the polymerase with ATP + CTP + GTP and with CTP + GTP + UTP,

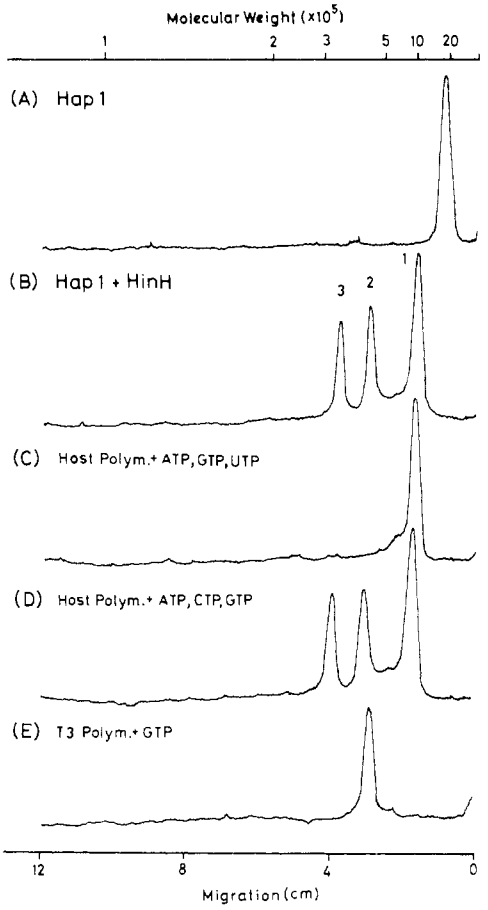

FIGURE 4: The binding of T3 and host RNA polymerases to the fragments produced from Hap 1 by the cleavage with HinH. The AGU fragments were prepared by binding to host polymerase as in the legend to Table III and resolved on 3% gels. The largest fragment (Hap 1) was extracted from the gels and digested with HinH. The digest was incubated with either host polymerase or T3 polymerase in the presence of indicated species of NTP. DNA fragments which formed complexes with the polymerases were isolated as in the legend to Table III. The fragments were layered on 5% gel columns and electrophoresed for 16 hr at 2 mA/tube. The autoradiographs were taken, and the density was traced.

suggesting that the AGU fragments might contain additional binding sites where the binding of the polymerase is stabilized with the other combinations of NTP's. Note that, because the AGU fragments were not recovered with ATP + CTP (Table III), we assume that the binding of RNA polymerase to DNA occurs at specific sites depending on the combination of NTP's.

Gel Electrophoresis of DNA Fragments which Bind to RNA Polymerases. DNA fragments which formed stable complexes with RNA polymerases in the presence of NTP's were dissociated from the complexes and resolved on gel electrophoresis. As shown in Figures 2B–G, a limited number of fragments were detected. The patterns were reproducible and only dependent on the species of RNA polymerases and NTP's used for the isolation of fragments. It was noted, however, that in some cases the same fragments were recovered by different combinations of the polymerases and NTP's. For example, Hap 1 was bound to the host polymerase with ATP + CTP + GTP, ATP + GTP + UTP, and CTP + GTP + UTP, in addition to being obtained with T3 polymerase and GTP. The observation suggests that most DNA fragments isolated by the interaction with RNA polymerases contained several different binding sites along each strand.

As the host polymerase gave a relatively high background (DNA fragments retained on the filter without adding NTP), the fraction was recovered and analyzed by gel electrophoresis. However, no significant band was detected. As a significant amount of DNA fragments was bound to the host polymerase core enzyme, the fragments which formed stable complexes in the presence of ATP + CTP + GTP were also analyzed. The pattern obtained was essentially identical with that of the original Hap digest (Figure 2H), indicating that the selectivity of the binding sites by the polymerase was lost by removing the σ factor.

Physical Mapping of RNA Polymerase Binding Sites and RNA Initiation Sites on Hap 1. The correlation between the RNA polymerase binding sites and RNA initiation sites was analyzed using Hap 1. As Hap 1 appears to contain several different binding sites along the strand, this fragment was isolated and further cleaved into smaller pieces by two different restriction endonucleases, Hga and HinH. The size of Hap 1 was estimated to be about 1.9×10^6 daltons (Figure 4A). Treatment of this fragment with HinH yielded three fragments (HinH 1, HinH 2, and HinH 3), of which the sizes are roughly 1.1×10^6, 0.45×10^6, and 0.35×10^6 daltons, respectively (Figure 4B). When the affinity of these fragments to the polymerases was examined, HinH 2 was efficiently bound to the T3 polymerase with NTP mixtures containing GTP (Figure 4E). With the host polymerase, HinH 1 was recovered with ATP + GTP + UTP and all three fragments with ATP + CTP + GTP (Figure 4C,D).

Upon treatment of Hap 1 with Hga, ten fragments were produced (Hga 1 to Hga 10 in Figure 5). Among these fragments, Hga 1 was efficiently bound to the T3 polymerase with GTP present (Figure 5D). Hga 2, Hga 3, and Hga 4 (or 5) were recovered with the host polymerase and ATP + CTP + GTP (Figure 5B), and Hga 3 and Hga 4 (or 5) were recovered with the host polymerase and CTP + GTP + UTP (Figure 5C). However, no fragment was bound to the host polymerase with ATP + GTP + UTP, suggesting that the original site at which the binding of the polymerase was stabilized with ATP + GTP + UTP was damaged by the cleavage with Hga. These results indicate that Hap 1 contains at least one binding site for the T3 polymerase and six binding sites for the host polymerase: one dependent on ATP + GTP + UTP, two on CTP + GTP + UTP, and three on ATP + CTP + UTP.

As has been shown previously (Takeya and Fujisawa, 1973), the host polymerase predominantly initiates two species of RNA starting with pppAUG- - - and pppGU- - - at the closely spaced sites near the left-most end of T3 DNA and transcribes about 25% length of T3 DNA, yielding 37S RNA (Figure 6A). When the Hap digest of T3 DNA was used as template for the host polymerase reaction, both (pppA- - -)RNA and (pppG- - -)RNA yielded major peaks at the 17S region (Figures 6B,C). This size of RNA was also formed on the isolated Hap 1 (Figure 6D). As the efficiency of RNA initiation was not influenced by the digestion of T3 DNA with Hap (see Table I), the result indicates that Hap 1 contained the left end promoters. Note that since Hap 1 contained one of the termini of T3 DNA (see Figure 2A), this fragment is derived from the left-most end of T3 DNA. The (pppA- - -)RNA and (pppG- - -)RNA formed on Hap 1 appeared to have almost identical sizes because they were only slightly separated by

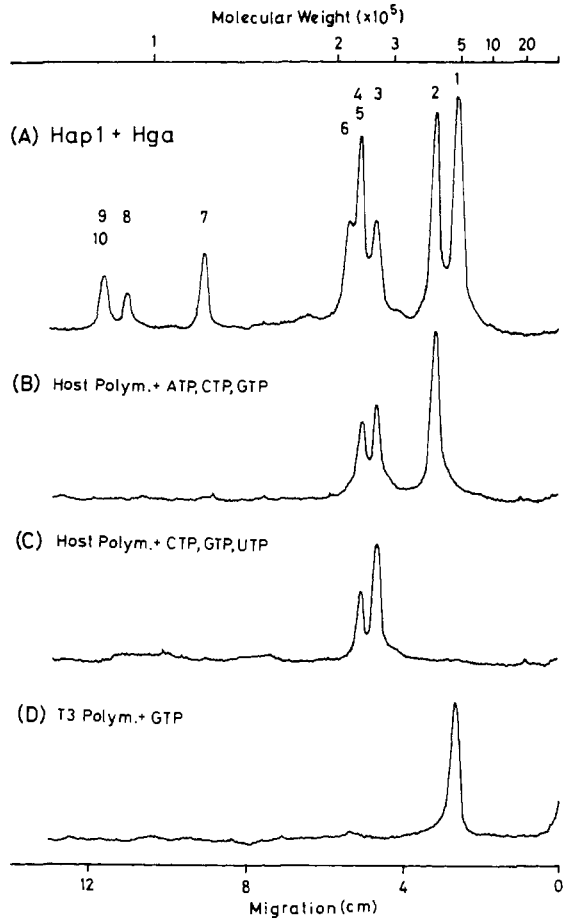

FIGURE 5: The binding of T3 and host RNA polymerases to the fragments produced from Hap 1 by the cleavage with Hga. Hap 1 was prepared as in the legend to Figure 4 and digested with Hga. The digest was incubated with either host polymerase or T3 polymerase in the presence of indicated species of NTP. DNA fragments which formed complexes with the polymerases were isolated as in the legend to Table III. The fragments were electrophoresed on 5% gels, and the resulting autoradiographs were traced.

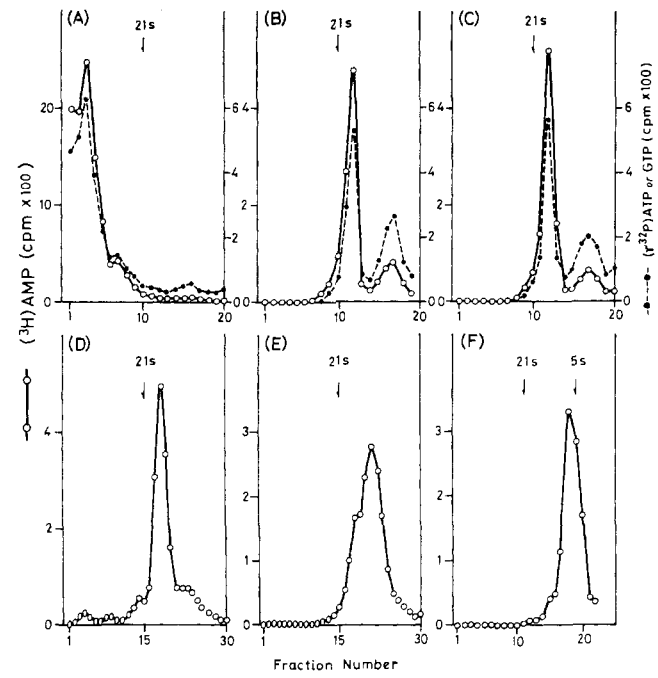

FIGURE 6: The size of RNA formed on the fragments produced from T3 DNA by the cleavage with restriction endonucleases. T3 DNA or its fragments were incubated for 20 min at 37° in RNA synthesizing mixtures containing [^{3}H]ATP (6000 cpm/nmol) and either host polymerase or T3 polymerase. When the starting termini were labeled, [γ-^{32}P]ATP or [γ-^{32}P]GTP (10^7 cpm/nmol) was added. Synthesized products were isolated, layered on sucrose density gradients, and centrifuged for 16 hr at 24,000 rpm. Hap 1 was prepared as in the legend to Figure 4. The approximate positions of fd RF-I DNA (21 S) and 5S ribosomal RNA as markers were indicated: (A) total RNA and [γ-^{32}P]ATP-RNA formed on intact T3 DNA by host polymerase; (B) total RNA and [γ-^{32}P]ATP-RNA formed on the Hap digest of T3 DNA by host polymerase; (C) total RNA and [γ-^{32}P]GTP-RNA formed on the Hap digest of T3 DNA by host polymerase; (D) total RNA formed on Hap 1 by host polymerase; (E) total RNA formed on the HinH digest of Hap 1 by host polymerase; (F) total RNA formed on Hap 1 by T3 polymerase.

running for long distances on gel electrophoresis. The size of the 17S RNA was estimated to be about 7×10^5 daltons, using an empirical equation in which ribosomal RNA, tRNA, and phage RNA fit (Boedtker, 1968). Therefore, the initiation sites for two RNA species would be at two closely spaced sites about 26% of the distance along the left end of Hap 1.

When the HinH digest of Hap 1 was used as template for the host polymerase, the size of the major RNA formed was about 12 S, corresponding to about 3×10^5 daltons (Figure 6E). Among the three fragments produced by the cleavage with HinH, the one which is long enough to form the 12S RNA would be HinH 1. The result also suggests that HinH 1 is located at the left end of Hap 1 (Figure 7). Hap 1 was treated with alkaline phosphatase and rephosphorylated with ^{32}P in the polynucleotide kinase reaction. Following the cleavage with HinH, ^{32}P was found at HinH 1 and HinH 2 (data are not shown). Therefore, it is reasonable to arrange the three HinH fragments as in Figure 7.

When Hap 1 was used as template for the T3 polymerase reaction, the RNA synthesized sedimented at about 6 S (Figure 6F). As both host and T3 polymerases transcribe T3 DNA in the same direction (left and right) (Dunn *et al.*, 1972), this result suggests that the T3 polymerase initiates RNA synthesis near the right end of Hap 1. HinH 2, which

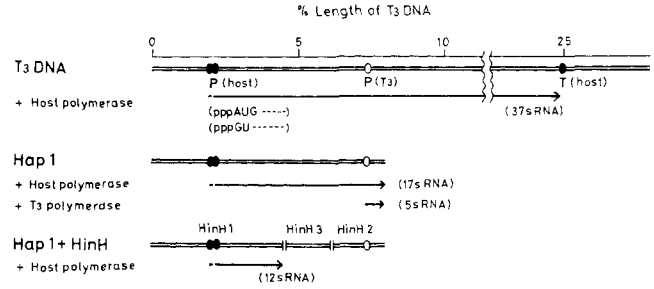

FIGURE 7: The cleavage sites and RNA initiation sites at the left-end region of T3 DNA: P, promoter; T, terminator.

was shown to be derived from the right end of Hap 1, had affinity toward the T3 polymerase (see Figure 4E). It is therefore likely that the T3 polymerase initiates RNA synthesis at its binding site (Figure 7).

Discussion

As a limited number of RNA polymerase molecules binds per DNA molecule in the absence of substrates (Hinkle *et al.*, 1972), the first step of RNA synthesis has been assumed to be the binding of RNA polymerase to the RNA initiation sites (promoters) on the template. In the present study, T3 DNA was cleaved into unique fragments by re-

striction endonucleases, and both the host and T3 polymerases were shown to bind to specific fragments depending on the combination of NTP's present in the binding mixture. The selectivity of binding by the host polymerase was lost if the σ factor was removed from the enzyme. These observations provide good evidence that the binding of RNA polymerases occurs at specific sites on the template.

The binding of RNA polymerase to DNA was greatly stabilized by the addition of NTP's in appropriate combinations. The apparent nucleotide K_m for polymerization has been estimated at about one-tenth of the K_m for the process of chain initiation (Anthony *et al.*, 1969). It is likely that the formation of oligonucleotides at RNA initiation sites results in an enhanced degree of polymerase stability on the DNA. Longer oligonucleotides appear to be required because the addition of one or two species of NTP's had little effect. Exceptions are for the binding of the T3 polymerase with GTP present and that of the host polymerase with ATP + CTP present. As the T3 polymerase has been shown to initiate RNA chains with pppGG- - - (Chakraborty *et al.*, 1973), it is likely that the binding of this enzyme is stabilized by the formation of G-oligonucleotides. Stabilization of the host polymerase binding with ATP + CTP is also probably explained by the formation of longer oligonucleotides consisting of only A and C.

The correlation between the RNA polymerase binding sites and RNA initiation sites was analyzed using the fragment derived from the left end of T3 DNA by the cleavage with Hap. This fragment (Hap 1) was further cleaved into smaller pieces with two other restriction endonucleases, and it was demonstrated that this fragment contains at least six different binding sites for the host polymerase and one binding site for the T3 polymerase. When this fragment was used as template for RNA synthesis, the T3 polymerase appeared to initiate RNA at the corresponding binding site. With the host polymerase, however, two species of RNA were predominantly synthesized on this fragment. The result suggests that RNA synthesis was efficiently initiated at two sites among the multiple binding sites detected. The mechanism that brings about the difference in efficiency of RNA initiation at different binding sites is not yet analyzed. There is the possibility that some of the binding sites detected are nonspecific binding sites with a structure similar to that of the promoter.

The procedure used in the present study should make possible isolation of DNA segments containing promoters from any DNA molecule. Although we used three species of restriction endonucleases for the cleavage of DNA, many bacterial strains are known to contain strain-specific enzymes. As each enzyme introduces duplex cleavages at different nucleotide sequences, a DNA fragment can be cleaved into smaller pieces by other enzymes with different cleavage site specificities. In this manner, it would be possible to isolate a short DNA segment containing an intact promoter. The procedure is also applicable to the physical mapping of RNA initiation sites. On the DNA of phage T7, closely related to T3, it has been shown that three species of early RNA are initiated near the left end of T7 DNA (Dunn and Studier, 1973). We have now mapped the RNA initiation sites at the left-end region of T3 DNA, and find that the T3 polymerase initiates RNA synthesis within the region transcribed by the host polymerase. This result is consistent with the hybridization experiment of Dunn *et al.*, (1972), which showed that part of the early RNA region of T3 DNA is transcribed by both the host and T3 polymerase.

References

Anthony, D. D., Wu, C. W., and Goldthwait, D. A. (1969), *Biochemistry 8,* 246.

Bigger, C. H., Murray, K., and Murray, N. E. (1973), *Nature (London), New Biol. 244,* 7.

Boedtker, H. (1968), *Methods Enzymol. 12B,* 429.

Boyer, H. W. (1971), *Annu. Rev. Microbiol. 25,* 153.

Boyer, H. W., Chow, L. T., Dugaiczyk, A., Hedgpeth, J., and Goodman, H. M. (1973), *Nature (London), New Biol. 244,* 40.

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

Chakraborty, P. R., Sarkar, P., Huang, H. H., and Maitra, U. (1973), *J. Biol. Chem. 248,* 6637.

Chamberlin, M. J., McGrath, J., and Waskell, L. (1970), *Nature (London) 228,* 227.

Dunn, J. J., Bautz, F. A., and Bautz, E. K. F. (1971), *Nature (London), New Biol. 230,* 94.

Dunn, J. J., McAllister, W. T., and Bautz, E. K. F. (1972), *Virology 48,* 112.

Dunn, J. J., and Studier, F. W. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1559.

Hedgpeth, J., Goodman, H. M., and Boyer, H. W. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 3448.

Hinkle, D. C., and Chamberlin, M. J. (1972), *J. Mol. Biol. 70,* 157.

Hinkle, D. C., Ring, J., and Chamberlin, M. J. (1972), *J. Mol. Biol. 70,* 197.

Kelly, T. J., Jr., and Smith, H. O. (1970), *J. Mol. Biol. 51,* 393.

Krakow, J. S., Daley, K., and Karstadt, M. (1969), *Proc. Nat. Acad. Sci. U. S. 62,* 432.

Maitra, U. (1971), *Biochem. Biophys. Res. Commun. 43,* 443.

Meselson, M., Yuan, R., and Heywood, J. (1972), *Annu. Rev. Biochem. 41,* 447.

Okamoto, T., Sugiura, M., and Takanami, M. (1972), *Nature (London), New Biol. 237,* 108.

Richardson, C. C. (1966), *J. Mol. Biol. 15,* 49.

Smith, H. O., and Nathans, D. (1973), *J. Mol. Biol. 81,* 419.

Sugisaki, H., and Takanami, M. (1973), *Nature (London), New Biol. 246,* 138.

Sugiura, M., Okamoto, T., and Takanami, M. (1969), *J. Mol. Biol. 43,* 299.

Takanami, M. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 34,* 318.

Takanami, M., Okamoto, T., and Sugiura, M. (1971), *J. Mol. Biol. 62,* 81.

Takeya, T., and Fujisawa, H. (1973), *Biochim. Biophys. Acta 324,* 110.

Travers, A. A., and Burgess, R. R. (1969), *Nature (London) 222,* 537.