Mapping of Class II Promoter Sites Utilized In Vitro by T7-Specific RNA Polymerase on Bacteriophage T7 DNA

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Restriction endonuclease *Bgl* II cleaves T7 DNA at a unique site (28.76% on the standard T7 map), yielding two fragments of molecular weights 18.9×10^6 (A) and 7.6×10^6 (B). Fragment B, representing the leftmost portion of the genome, has been purified by zone sedimentation. Transcription of fragment B by T7-specific RNA polymerase gives only r-strand-specific RNA. Analysis of the products by polyacrylamide gel electrophoresis reveals four major RNA species which have apparent molecular weights of 2.1×10^6 , 1.36×10^6 , 0.85×10^6 and 0.125×10^6 , respectively. Each of these RNAs is reduced in size when transcription is carried out with fragment B, which has been shortened by treatment with *Escherichia coli* exonuclease III. Therefore, each of the transcripts must be terminated at the right end of fragment B. Analysis of the molecular weights of the four transcripts produced from whole and from exonucleolytically shortened fragment B suggests that these transcripts are read from promoters located at 13.5, 18.9, 22.6, and 27.9%, respectively, on the standard T7 map. Hence, there are at least four promoters governing the transcription of the class II region. Transcripts initiated at these promoters on intact T7 DNA appear to read through the class II and part of the class III genetic region and terminate at the strong terminator for T7-specific RNA polymerase near 61%. Transcription of fragment B which has been cleaved with the restriction endonuclease *Hpa* I seems to activate a fifth promoter for T7-specific RNA polymerase. This promoter appears to be identical to the promoter previously described by Oakley and Coleman (Proc. Natl. Acad. Sci. U.S.A. 74:4266-4270, 1977) that maps near 15% on the standard T7 map. Little or no RNA is read from T7 *Bgl* II fragment B, which has a mobility expected for a transcript read from this promoter. However, upon cleavage with *Hpa* I, this promoter is utilized approximately 10-fold more efficiently than the other class II promoters. The mechanism of this activation is not yet known.

Bacteriophage T7 is a female-specific coliphage containing a linear, double-stranded DNA molecule with a molecular weight of 2.65×10^7 (20, 28). During the course of the 30-min infection cycle of T7 at 30°C, three temporal classes of T7-specific proteins can be observed on polyacrylamide gels (34). Class I proteins are those synthesized from 4 until 8 min after infection, class II proteins are those synthesized from 6 until 15 min after infection, and the class III proteins are those synthesized from 6 to 8 min after infection until lysis. The class I genes are clustered within the leftmost 20% of the T7 genome and are transcribed by the host RNA polymerase (15, 30). One of the products from the class I region of T7 is a new T7-specific RNA polymerase which transcribes the class II region (15 to 45% on the T7 genome) and the class III region (45 to 100% on the T7 genome) (4, 35).

Temporal control of T7 gene expression ap-

pears to be regulated primarily at the level of transcription (4, 14, 18). The cessation of class I gene expression involves both the phosphorylation of *Escherichia coli* RNA polymerase by a T7-induced protein kinase (41) and the binding of a T7-induced inhibitor protein to the former enzyme (12, 13). Processes which lead to cessation of class II transcription at about 15 min after infection are not well understood. However, T7-induced lysozyme may play a role (18).

Electrophoretic analysis of the RNAs transcribed by T7-specific RNA polymerase in vitro with T7 DNA as template gives six major size classes of RNA, which reflect RNA species read from seven transcription units located in the region from 45 to 100% on the standard T7 map (2, 7, 8, 22). Although no major transcripts from the class II region are observed on gels when whole T7 DNA is used as template, class II-specific RNA is being made. This has been

shown by electron microscopic examination of hybrids made between in vitro T7 RNAs and T7 DNA (32), in vitro translation of T7 RNAs (22; Golomb and Broach, unpublished data), and hybridization of T7 RNAs to *Hpa* I restriction fragments of T7 DNA which have been transferred to nitrocellulose filters by the procedure of Southern (19). Failure to detect class II transcripts as major transcription products is explained if class II promoters are utilized less efficiently than class III promoters (2). In addition, as we discuss below, transcripts which initiate at T7 class II promoters read to the T7 terminator near 61%. These transcripts have sizes that are very close to those of the major T7 transcripts I and II. If a class II promoter were utilized only 10% as often as a class III promoter, then the class II transcript would not normally be discerned above the background of the gel in the region of the major T7 transcripts I and II. Indeed, hybridization of the RNA produced in vitro by T7-specific RNA polymerase from whole T7 DNA to *Hpa* I restriction fragments of T7 DNA transferred to nitrocellulose filters by the Southern technique indicates that relatively little of the RNA made in vitro is derived from the class II region (19).

Recently, Oakley and Coleman (24) have mapped and sequenced a promoter for T7-specific RNA polymerase from the class II region of T7. The promoter was found to lie at the junction between genes 1 and 1.1 near 15% on the standard T7 map. The sequence of this region contains a high degree of symmetry.

To investigate further class II promoters in this region, we isolated a restriction fragment containing the leftmost 29% of T7 DNA, which contains none of the known, strong class III promoter sites for T7-specific RNA polymerase. Transcription of this fragment by T7-specific RNA polymerase gives rise to four discrete class II transcripts, and the promoter sites for these four transcription units have been mapped on the T7 genome.

MATERIALS AND METHODS

Chemicals. 32 P-labeled inorganic phosphate was purchased from New England Nuclear Corp. and was converted to α -[32 P]CTP by the method of Symons (37). Unlabeled nucleoside triphosphates were purchased from PL-Biochemicals and Calbiochem. Spermidine and dithiothreitol were purchased from Sigma Chemical Co. Sucrose (nuclease-free) was purchased from Bethesda Research Laboratories. Hydroxylapatite (Bio-Gel HTP) and low gel temperature agarose were purchased from BioRad Laboratories. Formamide (99%) was obtained from Eastman Organic Chemicals. Nitrocellulose sheets (0.45 μ m, BA 85) were purchased from Schleicher & Schuell. Polymyxin P (polyethyleneimine) was obtained from BASF and was

prepared as a 10% solution and neutralized with HCl. Heparin coupled to cyanogen bromide-activated Bio-Gel A50M was the gift of B. Davison.

Nucleases. Exonuclease III was the generous gift of C. C. Richardson (Harvard Medical School). *Aspergillus oryzae* S1 nuclease was the gift of P. Berg (Stanford Medical School). The restriction endonucleases *Dpn* II and *Hpa* I were prepared by T. Kadesch, and the restriction endonuclease *Bgl* II was purified by J. Clark in this laboratory by a modification of an unpublished procedure of R. Roberts (Cold Spring Harbor Laboratory). A 1- μ l amount of the latter enzyme preparation will cleave 15 μ g of T7 DNA to completion in 8 h at 37°C and contains no apparent contaminating endonuclease or exonuclease activity. Pancreatic RNase A was purchased from Worthington Biochemicals Corp.

Purification of T7-specific RNA polymerase. DG 156, an RNase I-deficient derivative of *E. coli* B/1 (7), was grown at 30°C to a cell density of 10^{10} cells per ml in 200 liters of minimal medium (M56 [40]) containing 2% glucose in a New Brunswick Scientific Fermacell 250 fermentor and was infected with T7 at a multiplicity of infection of 6. At 9 min after infection, the cells were chilled and harvested by centrifugation. The cells were then resuspended in an equal volume of 50 mM Tris-hydrochloride (pH 8.0)-10% sucrose and were stored at -70°C.

T7-specific RNA polymerase was purified by a procedure that utilized the polyethyleneimine precipitation step of Niles et al. (23) for the removal of nucleic acids. A summary of the purification up to fraction IV is presented in Table 1. All operations were carried out at 0 to 2°C, and all centrifugations were carried out in a Sorvall GSA rotor. Frozen cells (800 g) were suspended in 50 mM Tris-hydrochloride (pH 8.0)-10% sucrose (1,200 ml), and 0.1 M $\text{Na}_3\text{-EDTA}$ and 0.1 M dithiothreitol were added to give final concentrations of 2 and 0.1 mM, respectively. Solid spermidine was then added to give a final concentration of 7 mg/ml, followed by addition of lysozyme to give a final concentration of 150 μ g/ml. After 30 min, a 10% solution of sodium deoxycholate was added to give a final concentration of 0.05%, and incubation was continued for 15 more min. The cell lysate was centrifuged for 50 min at 15,000 $\times g$, and glycerol was added to the supernatant fluid to give a final concentration of 5% (fraction I).

TABLE 1. Purification of T7-specific RNA polymerase to fraction IV

Fraction	Vol (ml)	Total activity (units $\times 10^{-3}$)	Protein (mg/ml)	Sp act (units/mg)	% Total activity
I. Crude extract	1,700	14,500	15.8	540	100
II. Polyethyleneimine pool	1,070	9,300	6.1	1,430	64
III. $(\text{NH}_4)_2\text{SO}_4$ precipitate	1,080	9,000	3.7	2,240	62
IV. Phosphocellulose					
Pooled fractions I	9	2,800	2.9	104,000	28
Pooled fractions II	7.5	1,200	3.7	47,000	

A 10% solution of polyethyleneimine was added to fraction I slowly with stirring to give a final concentration of 1.2%, and stirring was continued for 30 min. The resulting precipitate was collected by centrifugation at $15,000 \times g$ for 30 min. The supernatant fluid, which contained insignificant amounts of T7-specific RNA polymerase activity, was discarded, and the precipitate was resuspended in 800 ml of buffer (10 mM Tris-hydrochloride [pH 8]–0.1 mM dithiothreitol–5% glycerol–0.5 M NH_4Cl). After stirring for 15 min, the suspension was centrifuged at $10,000 \times g$ for 20 min, and the supernatant fluid was saved. The pellet was resuspended in 300 ml of the same buffer and centrifuged, and this supernatant fluid was combined with the previous one (fraction II). Solid, ultrapure ammonium sulfate (0.36 g/ml) was added to fraction II slowly with constant stirring, and after stirring 40 min the precipitate containing the T7-specific RNA polymerase was collected by centrifugation ($10,000 \times g$ for 30 min). The precipitate was then dissolved in 1,000 ml of a solution containing 10 mM Tris-hydrochloride (pH 8)–0.1 mM dithiothreitol–5% glycerol (fraction III).

Fraction III (1,080 ml) was passed through a column of phosphocellulose P-11 (2.5-cm diameter by 18-cm length) and was washed with 250 ml of a buffer containing 20 mM sodium phosphate (pH 7.8)–0.1 mM dithiothreitol–0.1 mM $\text{Na}_3\text{-EDTA}$ –10% glycerol (buffer P) and 0.2 M NaCl. The T7-specific RNA polymerase was eluted from the column with buffer P–0.6 M NaCl. Fractions containing 70% of the activity were combined into two pools and were dialyzed against buffer P containing 50% glycerol (fraction IV). T7-specific RNA polymerase preparations at this stage are stored at -20°C and have retained 80 to 100% of their original activity after 2 years.

Fraction IV enzyme was purified further by chromatography on heparin-agarose. A sample of fraction IV (pool I; 2 mg in 0.7 ml) was passed through a heparin-Bio-Gel A50M column (1-ml total volume), and the column was washed with 5 ml of buffer P. The enzyme was eluted with a linear gradient from 0 to 0.5 M NaCl in buffer P (20 ml). T7-specific RNA polymerase activity appeared as a single peak at a conductivity equivalent to buffer P + 0.4 M NaCl. Peak fractions (containing 67% of the original activity) were pooled and were concentrated by dialysis against buffer P containing 50% glycerol. This dialysis resulted in a substantial drop in the specific activity of the enzyme preparation from 650,000 to 390,000 U/mg, and alternate methods of concentration should probably be explored.

T7-specific RNA polymerase assay. During the purification of the enzyme, T7-specific RNA polymerase was assayed as previously described (5). In subsequent studies, a reaction solution (100 μl) containing 40 mM Tris-hydrochloride (pH 8); 8 mM MgCl_2 ; 5 mM dithiothreitol; 4 mM spermidine; 0.4 mM ATP, GTP, and UTP; $\alpha\text{-}^{32}\text{P}$ CTP (5 to 1,000 cpm/pmol); and 0.3 mM T7 DNA was employed. Each reaction contained 1 to 20 U of T7-specific RNA polymerase, where 1 U is a rate of 1 nmol of CMP incorporated per h as determined in a 10-min assay at 37°C . Replacement of serum albumin with spermidine (4 mM) gives nearly optimal T7-specific RNA polymerase activity, but

eliminates the problems caused by the contamination of serum albumin by RNase. In the presence of 4 mM spermidine, the MgCl_2 optimum for T7-specific RNA polymerase is reduced to 8 mM MgCl_2 .

Purification of T7 *Bgl* II fragment B. T7 DNA was isolated from purified phage by phenol extraction (38). Cleavage of T7 DNA was carried out in a reaction (2.5 ml) containing 6 mM Tris-hydrochloride (pH 8), 6 mM MgCl_2 , 6 mM β -mercaptoethanol, 100 μg of gelatin per ml, 0.4 mg of T7 DNA per ml and 80 μl of our endonuclease *Bgl* II preparation. After 8 h at 37°C , the DNA was deproteinized by phenol extraction. Samples containing 50 μg of *Bgl* II-cleaved T7 DNA were layered onto 12.4-ml sucrose gradients (10 to 30%) containing 10 mM Tris-hydrochloride (pH 8)–1 mM $\text{Na}_3\text{-EDTA}$ and were sedimented at 40,000 rpm for 15 h in a Beckman SW41 Ti rotor at 5°C . Fractions (0.3 ml) were collected from the bottom of each gradient, and even-numbered fractions were analyzed by electrophoresis on 0.7% agarose slab gels (9 by 9 cm by 3 mm) with the Tris-acetate buffer system described by Hayward and Smith (11). Fractions containing primarily T7 *Bgl* II fragment B were pooled, concentrated by chromatography on hydroxylapatite, and finally dialyzed into a solution containing 10 mM Tris-hydrochloride (pH 8)–1 mM $\text{Na}_3\text{-EDTA}$. These fractions were sedimented once more on 10 to 30% sucrose gradients and then were collected and analyzed as before. Fractions containing only T7 *Bgl* II fragment B were pooled, concentrated on hydroxylapatite, and finally dialyzed into a solution containing 10 mM Tris-hydrochloride (pH 8)–0.1 mM $\text{Na}_3\text{-EDTA}$. The final fragment B preparation was relatively free of detectable single-strand breaks when analyzed by electrophoresis in alkaline agarose gels (20) and stained with acridine orange (21). When large amounts of fragment B were analyzed by agarose gel electrophoresis, trace amounts of fragment A could be observed; however, none of the known class III transcripts was detected in the RNA product either by gel electrophoresis or by hybridization to purified T7 *Bgl* II fragment A.

Isolation of separated transcripts and hybridization to DNA fragments on nitrocellulose filter strips. When transcripts were to be isolated for hybridization, the T7 RNA products were fractionated by electrophoresis through slab gels (7) containing 1.4% low gel temperature agarose. The individual transcripts were located by autoradiography, excised from the gel, and placed into small polystyrene tubes. The agarose was melted in a 65°C water bath, and the samples were loaded onto 0.5-ml hydroxylapatite columns which were kept at 37°C in an incubator oven. Each column was washed with 2 ml of 50 mM potassium phosphate (pH 7.8), and the RNA was eluted with 0.3 M potassium phosphate (pH 7.8). Formamide (99%) was added to the eluted RNA (volume $\approx$ 1 ml) to give a final concentration of 50%.

T7 *Bgl* II fragment B was cleaved with *Hpa* I under the same reaction conditions used for *Bgl* II cleavage. Samples (0.2 μg) of the *Hpa* I-cleaved T7 *Bgl* II fragment B were loaded on a 0.8% agarose gel and subjected to electrophoresis for 10 h at 40 mA on a horizontal gel apparatus similar to that described by McDonnell et al. (20). The *Hpa* I fragments in the

agarose gel were denatured and transferred to a sheet of nitrocellulose filter paper by a modification of the procedure of Southern (17).

The nitrocellulose filter was cut into strips containing separate tracks of the agarose gel and was incubated with the isolated RNA in 50% formamide to allow hybridization. This was accomplished by incubating the two components in a heat-sealed plastic cooking pouch for 18 h in a 37°C water bath. The filter strips were then removed from the pouches, were each rinsed with 50 ml of 5× SSC, and were then incubated in 50 ml of 4× SSC–50% formamide for 2 h at 37°C. This solution was decanted off, and the filters were then incubated with 50 ml of 2× SSC containing 20 µg of RNase A per ml for 1 h at room temperature. The filters were finally washed by vigorous shaking in 5× SSC for 30 min and were then air dried on a sheet of Saran wrap. Hybridized, labeled RNA was detected by autoradiography at –70°C with Kodak XR-5 film and a du Pont Cronex X-ray screen.

Analytical gel electrophoresis of RNA transcripts. RNA transcripts from in vitro synthesis reactions were analyzed by electrophoresis of samples (50,000 to 100,000 cpm) on 2.0% acrylamide–0.5% agarose gels as described by Golomb and Chamberlin (8) followed by autoradiography. For this gel system there is an inverse linear relationship between the logarithm of the molecular weight of an RNA and the mobility of that species over the range from about 10^5 to 3×10^6 (M. Golomb, Ph.D. thesis, University of California, Berkeley, 1974).

RESULTS

T7 DNA is cleaved by the restriction endonuclease *Bgl* II at one site, producing two fragments (Fig. 1, track b). *Bgl* II cuts between the A and the G in the 5' → 3' sequence AGATCT (27). Because the internal four nucleotides of this sequence, GATC, constitute the recognition and cleavage site for the restriction endonuclease *Dpn* II, it necessarily follows that any *Bgl* II cleavage site is also a *Dpn* II cleavage site. To map the cleavage site of *Bgl* II on T7 DNA it was only necessary to compare the *Bgl* II restriction fragments with a partial digest of T7 DNA by *Dpn* II because an accurate map for the *Dpn* II cleavage sites had already been obtained by McDonnell et al. (20). Figure 1, track a, shows that the smaller of the two *Bgl* II fragments (fragment B) has the same migration rate as the *Dpn* II partial cleavage product which contains *Dpn* II fragments B, G, and D. This result implies that *Bgl* II cleaves T7 DNA at the *Dpn* II site positioned at 28.76% on the standard T7 map.

The finding that *Bgl* II cleaves T7 DNA at one site near 29% made it clear that T7 *Bgl* II fragment B would be a very useful template for the study of T7 class II promoters in vitro, because it contains the left half of the class II region and eliminates competition from the class III promoters (see Fig. 6 below). Because T7 *Bgl*

FIG. 1. Restriction analysis of *Bgl* II fragment B on a 0.7% agarose gel. (a) and (e), 0.4 µg of T7 DNA partially digested with *Dpn* II; (b), 0.39 µg of T7 DNA digested with *Bgl* II; (c), 0.1 µg of final *Bgl* II fragment B preparation; (d), 0.1 µg of *Bgl* II fragment B digested with *Dpn* II. The identification of the *Dpn* II restriction fragments is given at the left of the figure.

II fragment A is nearly 2.5 times the size of fragment B, it was possible to separate the two fragments by sedimentation through sucrose gradients. Figure 2 shows the separation of T7 *Bgl* II fragments A and B on a single 10 to 30% sucrose gradient. When the pooled fractions containing primarily fragment B were sedimented on another 10 to 30% gradient, a nearly homogeneous preparation of fragment B was obtained (Fig. 1, track c). Digestion of purified T7 *Bgl* II fragment B with the restriction endonuclease *Dpn* II gave rise to T7 *Dpn* II fragments B and D (Fig. 1, track d) as predicted.

When T7 *Bgl* II fragment B was used as a template for T7-specific RNA polymerase, maximal activity was observed at template concentrations near 0.06 mM (in nucleotides), in contrast to the 0.2 mM template concentration required for whole T7 DNA to obtain maximal activity. Even at these optimal template concentrations, however, the total rate of RNA synthesis by T7-specific RNA polymerase with the T7 *Bgl* II fragment B was only one third of that obtained with intact T7 DNA. This is probably a consequence of the fact that T7 *Bgl* II fragment B possesses only the weak class II T7 promoters which are read more slowly than the strong class III promoters which are present on intact T7 DNA. Over 97% of the RNA made by T7-specific RNA polymerase with T7 *Bgl* II fragment B as template was hybridizable to the r strand of T7 DNA. This result is in agreement with the original observation that all transcription by T7-specific RNA polymerase on T7 DNA is r strand specific (4, 36).

When the T7-specific RNA polymerase transcription products of T7 DNA which had been cleaved with *Bgl* II (where fragments A and B are not separated) were analyzed by polyacryl-

FIG. 2. Sedimentation of *Bgl* II-cleaved T7 DNA on the first 10 to 30% sucrose gradient. Aliquots (10 μ l) from even fractions 2 to 40 (left to right) were subjected to electrophoresis on a 0.7% agarose gel (see text for details). Fractions 15 to 19 were pooled for resedimentation.

amide gel electrophoresis, two new transcripts were observed along with the expected six major size classes of class III transcripts (Fig. 3, track b). Because these two new RNA species, designated VIII* and X*, were not observed without *Bgl* II cleavage, they probably arise from transcripts initiated at two separate promoters and elongated to the right end of *Bgl* II fragment B. (We designate the T7 RNA transcripts from T7 *Bgl* II fragment B as RNA species VII* to X* in order of decreasing molecular weight, and the corresponding transcripts read from intact T7 DNA would be T7 RNA species VII to X. This nomenclature differs slightly from the nomenclature used by Golomb and Chamberlin [9] and Beier et al. [2]. In these papers, minor RNA species of high molecular weight, resulting from the readthrough of RNA species III, IV, and V past the 61% terminator, were designated RNA species VII, VIII, and IX, respectively. We feel it is more appropriate to let different Roman numerals represent transcripts from different promoters, read to the first strong terminator, and use an alternative nomenclature for read-through transcripts. By this nomenclature T7 RNA species VIII, for example, is read from the promoter at 18.9% through to the strong terminator near 61%.) Analysis of the products transcribed from purified T7 *Bgl* II fragment B showed that along with RNA species VIII* and X*, two other transcripts were made from this fragment (RNA species VII* and IX*) (Fig. 3, track c). T7 *Bgl* II fragment B RNA species VII*, VIII*, IX*, and X* have apparent molec-

ular weights of 2.1×10^6 , 1.36×10^6 , 0.85×10^6 , and 0.12×10^6 , respectively, when T7 RNA species I through VI serve as molecular-weight standards (7).

It is most plausible that the four class II transcripts read from T7 *Bgl* II fragment B are initiated at four independent promoter sites in the class II region of the fragment and are each terminated at the right end of fragment B. To test this possibility, T7 *Bgl* II fragment B was enzymatically shortened by treatment with exonuclease III and S1 nuclease (7). The extent of exonuclease truncation was monitored quantitatively by cleaving the exonucleolytically shortened T7 *Bgl* II fragment B with *Dpn* II and analyzing the amount of shortening of T7 *Dpn* II fragments B and D by agarose gel electrophoresis (Fig. 4a and b). The major source of error in measuring the extent of exonuclease digestion recorded in Table 2 is due to the distortion of the DNA bands in the agarose gel (Fig. 4a).

Figure 5 shows an autoradiogram of the RNA made by T7-specific RNA polymerase on T7 *Bgl* II fragment B (track b) and T7 *Bgl* II fragment B which had been treated with exonuclease III for 3 min (track c) and 6 min (track d). Exonucleolytic treatment of *Bgl* II fragment B causes the shortening of RNA species VII*, VIII*, and IX* and the disappearance of RNA species X*. The amount of shortening of each of the individual RNA species is the same and is equal to the amount of shortening of the fragment-B DNA template (Table 2). Therefore, the apparent molecular weights of these RNA species are con-

FIG. 3. Electrophoretic analysis of the transcripts made by T7-specific RNA polymerase from Bgl II-cleaved T7 DNA and Bgl II fragment B. Reaction conditions were as described in the text where 0.03 μ g of T7-specific RNA polymerase was added to start RNA synthesis. Samples were analyzed by electrophoresis on 2.0% acrylamide-0.5% agarose gels, followed by autoradiography (8). 0.3 mM T7 DNA (a and d), 0.24 mM T7 DNA cleaved with Bgl II (b), and 0.05 mM Bgl II fragment B (c) were used as templates.

FIG. 4. Digestion of *Bgl* II fragment B with exonuclease III and S1 nuclease. (a) 0.7% agarose gel analysis of the extent of exonuclease digestion. *Dpn* II-cleaved T7 DNA, tracks (a) and (d), and *Dpn* II-cleaved T3 DNA, track (e), were used as molecular weight standards. Track (b), *Bgl* II fragment B digested for 3 min and then cleaved with *Dpn* II; track (c), *Bgl* II fragment B digested for 6 min and cleaved with *Dpn* II. (b) Plot of the extent of exonuclease digestion of *Bgl* II fragment B in standard T7 physical map units versus time. Symbols: ●, percent decrease in *Dpn* II fragment B; ○, percent decrease in *Dpn* II fragment D.

sistent with the published molecular weights for T7 *Dpn* II fragments B and D (20). Quantitatively, the results of this experiment also support the notion that the rate of migration of the four *Bgl* II fragment B transcripts in the gel electrophoresis system that we have used gives a fairly accurate measure of their true molecular weights. We conclude that RNA species VII* to X* are derived from four separate promoters and terminate at the right end of fragment B.

From average apparent molecular weights of the four class II transcripts calculated from a number of different gel experiments and from the results of the exonuclease experiment shown in Table 2, we position the four class II promoters VII to X at 13.5, 18.9, 22.6, and 27.9%, respectively, on the standard T7 map (Fig. 6).

Oakley and Coleman (24) have isolated two T7 *Hpa* II restriction fragments (*Hpa* II-138 and *Hpa* II-140) that each contain a promoter for T7-specific RNA polymerase. Both fragments were found to be contained within T7 *Hpa* I fragment G (Fig. 6) in the class II region of T7. Fragment *Hpa* II-138 was sequenced, and its promoter for T7-specific RNA polymerase was positioned near 15% on the T7 map. *Hpa* II-140 appears to map directly to the left of *Hpa* II-138 on T7, positioning its promoter at approximately 14% (24). The RNA species VII promoter that we have observed and mapped at 13.5% on T7 probably corresponds to the promoter that Oakley and Coleman have observed on *Hpa* II-140. However, we have not observed a transcript made from T7 *Bgl* II fragment B of a size that could arise from a promoter near 15% on the T7 genome.

To explore the question of the 15% T7 class II promoter in more detail, we have cleaved T7 *Bgl* II fragment B with the restriction endonuclease *Hpa* I before using these fragments as templates for T7-specific RNA polymerase. *Hpa* I cleaves T7 *Bgl* II fragment B at four sites (20) (see Fig. 6), giving rise to *Hpa* I fragments F, H, G, Q, and a slightly shortened C. Rather surprisingly, cleavage of T7 *Bgl* II fragment B by *Hpa* I leads to a 40% increase of T7-specific RNA polymerase activity with the fragments as template. An autoradiogram of the reaction products made from *Hpa* I-cleaved T7 *Bgl* II fragment B is shown in Fig. 7, track b. A number of minor transcripts are observed, and these probably result from end-to-end transcription of the *Hpa* I fragments, a phenomenon originally reported by Oakley and Coleman (24). However, the most striking result of this experiment is the appearance of a new transcript among the RNA products (designated Y) of molecular weight 0.5×10^6 . The promoter for this transcript appears to be utilized at least 10 times more efficiently than the RNA species IX promoter. (RNA species corresponding to transcripts from promoters VIII and X are not seen in this experiment because they are too short to remain on this gel.)

To determine the origin of the Y transcript, this RNA was isolated as described above and was hybridized to *Hpa* I fragments of *Bgl* II fragment B which had been transferred to nitrocellulose filters by a modification of the proce-

TABLE 2. Reduction in the size of transcripts from exonucleolytically shortened *Bgl* II fragment B^a

Duration of exonuclease III treatment (min)	% Reduction in template strand relative to whole T7 DNA	% Reduction in transcripts relative to whole T7 DNA			
		RNA species VII	RNA species VIII	RNA species IX	RNA species X
3	2.5 ± 0.3	2.35 ± 0.15	2.25 ± 0.3	2.25 ± 0.3	— ^b
6	5.1 ± 0.3	5.1 ± 0.3	5.3 ± 0.5	ND ^c	— ^b

^a The extent of exonuclease digesting and the reduction in the size of the RNA transcripts were derived from Fig. 4 and 5. The amount of RNA and DNA shortening was defined in terms of percentage of units of a whole T7 DNA molecule containing 40,000 base pairs (20, 28). The error in the size of the RNA molecules was estimated from their band widths on gels.

^b —, RNA species absent or unidentifiable.

^c ND, RNA species present, but size not determined.

ture of Southern (17). Nearly all of the labeled RNA was found to hybridize in the region of *Hpa* I fragments F and G (Fig. 8). Hence, transcript Y most likely originates from a promoter in fragment G and terminates at the right end of fragment G. Transcript Y cannot originate from fragment F because the Y transcript is unaltered in molecular weight and is observed as the major transcription product from an *Hpa* I digest of T7 *Bgl* II fragment B containing a 2.2% deletion that removes the *Hpa* I cleavage site between F and H (data not shown). Skare et al. (32) have also shown that T7-specific RNA polymerase does not make RNA complementary to the region of T7 corresponding to *Hpa* I fragments F and H. From the apparent molecular weight of transcript Y, it can be calculated that the promoter for this transcript on *Hpa* I fragment G would be located at a site near 14.7% on intact T7 DNA. Hence, it is highly likely that this is the promoter observed by Oakley and Coleman (24) near 15% on the T7 genome. It appears that cleavage of T7 DNA with restriction endonuclease *Hpa* I activates a previously silent promoter.

DISCUSSION

The restriction endonuclease *Bgl* II cleaves T7 DNA at a single site near 29%. Transcription of purified T7 *Bgl* II fragment B (containing the leftmost 29% of T7 DNA) by T7-specific RNA polymerase reveals that there are at least four in vitro class II promoters. Because T7 *Bgl* II fragment B contains the left half of the class II region of T7, these four promoters probably represent the majority of class II promoters. Occasionally, we have observed a faintly labeled transcript with a molecular weight of approximately 3.6×10^6 when whole T7 DNA is used as a template for T7-specific RNA polymerase. This transcript is not affected by cleavage of T7 DNA with *Bgl* II. It is possible that this RNA originates from another, very weak class II promoter located near 35% on the standard T7 map. The results of Pachl and Young (26) suggest that

there is a late promoter near 35% on T7 which is utilized in vivo.

Transcripts initiated at these class II promoter sites on intact T7 DNA probably end at the strong terminator for T7-specific RNA polymerase which maps near 61% and which also serves to terminate the T7 class III transcripts initiated at promoters IIIa, IV, and V (2, 19). The molecular weights expected for the resulting primary class II transcripts read from intact T7 DNA are summarized in Table 3. It might be supposed that there is yet another terminator near 45% on the T7 genome which serves to terminate the class II transcripts at the end of the class II region. However, this possibility can be ruled out because the class II transcripts read from intact T7 DNA would migrate in a molecular weight region on gels (4×10^6 to 2.3×10^6) in which no transcripts are detected (Fig. 6). Even slight amounts of these transcripts would be discerned above the background in this region of the gel. Further evidence that the class II transcripts are read to the T7 terminator near 61% when intact DNA serves as template also comes from the observation that class II transcripts have molecular weights of over 4.5×10^6 when in vitro transcription products separated on gels are hybridized to restriction fragments (19) and when class II RNAs are transcribed from T3 × T7 recombinant phage (2).

Transcription of the class III region of T7 is characterized by the presence of two clusters of overlapping transcription units (Fig. 6). From our work and the in vivo studies of others (10, 25, 26), it appears that the class II region of T7 is also read from overlapping transcription units. Similar overlapping transcription units are seen for the related phage T3 (9) and for unrelated phages such as SP6 (E. T. Butler and M. Chamberlin, manuscript in preparation), ϕ X174 (1), and fd (29). This suggests that there is a strong selection for this kind of transcriptional map. At least one advantage of such an arrangement is that transcription of some genes is enhanced

FIG. 5. Electrophoretic analysis of the T7-specific RNA polymerase transcripts made from exonucleolytically shortened Bgl II fragment B. Samples were

relative to others, which may have significance in the quantitative regulation of gene expression.

There is evidence that T7 RNAs are transcribed *in vivo* from overlapping transcription units as well. Class III RNA species I to V can be observed on polyacrylamide gels of labeled RNAs extracted from RNase III⁻ cells infected with T7, where processing of the primary T7 transcripts does not occur (6). Multiple promoters also appear to be utilized from the transcription of the class II region *in vivo*, giving rise to a series of overlapping transcripts which are in turn processed by RNase III. Pachl and Young (26) have located four probable class II promoters to the left of 30% that are utilized *in vivo*, and these appear to correspond to the four *in vitro* class II promoters mapped in the current work. Their results also suggest that there are two additional class II promoters to the right of 30% on the T7 genome.

Oakley and Coleman have observed two promoters for T7-specific RNA polymerase which they positioned near 14 and 15%, respectively, in the region where we have positioned the promoter for RNA species VII (24). The latter site has been located more accurately at 14.6% by Studier (personal communication). Because we have observed only one promoter in this region of T7 when T7 Bgl II fragment B is used as template, it is plausible that the promoter that we observe at 13.5% corresponds to the promoter that Oakley and Coleman observed, located near 14% on T7. This does not explain why we do not see a separate transcript from T7 Bgl II fragment B read from the 14.6% promoter of Oakley and Coleman, although we discuss one possibility below. An alternative possibility is that RNA species VII* is read from a promoter at 14.6% and not from 13.5% (or 14%) but that our determination of the molecular weight of RNA species VII* is inaccurate. The precision of locating transcript VII* on the polyacrylamide gel is comparable to an uncertainty in molecular weight of about $\pm 50,000$, whereas the difference in molecular weight for RNAs read from 13.5 and 14.6% on T7 Bgl II fragment B is computed to be 150,000. Hence, the difference in molecular weight for transcripts read from these two sites is within the precision of our determination. However, the uncertainty in the size of T7 RNA species IIIa,b, which was used as a molecular

analyzed by electrophoresis on 2.0% acrylamide-0.5% agarose gels, followed by autoradiography (8). A 0.03- μ g amount of T7-specific RNA polymerase was added to reaction solutions containing T7 DNA (a and e), Bgl II fragment B (b), Bgl II fragment B digested for 3 min (c), and Bgl II fragment B digested for 6 min (d).

FIG. 6. Map of the *in vitro* transcripts formed by T7-specific RNA polymerase on T7 DNA. Distances indicated by heavy lines on the genetic map show the approximate size of the numbered T7 genes as calculated from the sizes of their known gene products (34). Genes for which the size of the product is not known are indicated by wavy lines. Positions of the genes are assigned on the basis of data presented by Simon and Studier (31), Hyman *et al.* (16), and Campbell *et al.* (3). The gene for DUP (DNA unwinding protein) has not been unambiguously mapped, and its position on this map is only tentative. The Dpn II and Hpa I restriction maps of T7 are from McDonnell *et al.* (20). The positions of promoter and terminator sites for the T7-specific RNA polymerase were estimated from the molecular weights of transcripts from exonucleolytically shortened templates (7; this work), analysis of the transcription pattern of T3 $\times$ T7 recombinant phages (2), and the hybridization studies of McAllister and McCarron (19).

weight standard, is $\pm 200,000$ (8), and as a result we cannot rule out the possibility that T7 RNA species VII* is actually initiated at 14.6%. Yet a third possibility is that transcripts are read from both the 13.5 and the 14.6% promoter on T7 *Bgl* II fragment B, but that they are not clearly resolved in our experiments from transcript VII* originating from the 13.5% promoter because the molecular weight difference between the two resulting transcripts is only 150,000; however, the band width of species VII* (cf. Fig. 3, track c, or Fig. 5, track b) is not detectably broader than that of other single RNA species such as VIII* or IX*, as might be expected if two RNA species were present. In addition species VII* does not split into two bands or become appreciably broader as its molecular weight is reduced by exonucleolytic shortening of the template (cf. Fig. 5, tracks c and d).

Whether the 14.6% promoter site is used weakly or not at all when intact T7 *Bgl* II fragment B serves as template, efficient transcription from this promoter depends upon cleavage of the DNA with restriction endonuclease *Hpa*.I. After cleavage with *Hpa*.I, this promoter is utilized about 10 times more efficiently than any of the other class II promoters, and it is attractive to assume from Oakley and Coleman's results that *Hpa*.II cleavage also activates this site. The observation that a previously silent or weak promoter site for T7-specific RNA polymerase is activated by restriction cleavage of the DNA template poses a potentially serious

problem for those who wish to use such fragments of DNA to map promoter and terminator sites. The phenomenon is surprising, because T7-specific RNA polymerase is less susceptible to initiating nonspecific RNA chains at breaks or ends as compared with the bacterial RNA polymerase (5), although there is some transcription from the ends of *Hpa*.II fragments (24) and *Hpa*.I fragments (Fig. 7).

We have not found any satisfactory explanation for the activation of the 14.6% promoter and stimulation of total RNA synthesis after *Hpa*.I cleavage of T7 DNA. The actual promoter sequence is located at least 1,000 nucleotides from the ends of the fragment. Hence, given the size of T7-specific RNA polymerase, it is unlikely that the 14.6% promoter is being read by T7-specific RNA polymerase which binds at the ends of the fragment. Nevertheless, because T7-specific RNA polymerase appears to bind to *Hpa*.I ends, it is possible that the proximity of the ends of *Hpa*.I-G fragment to the 14.6% promoter leads to a concentration of T7-specific RNA polymerase in that region which greatly enhances the utilization of that promoter. This model would not explain the activation of a minor promoter for T7-specific RNA polymerase which occurs when DNA from the T3 $\times$ T7 recombinant phage R8 is enzymatically shortened, a phenomenon which may be related to the activation of the 15% promoter discussed above (33). Recombinant phage R8 has only two strong promoters used by T7-specific RNA po-

FIG. 7. Electrophoretic analysis of the T7-specific RNA polymerase transcription product from *Hpa* I-cleaved *Bgl* II fragment B. T7-specific RNA polymerase reaction products from whole T7 DNA (0.3 mM, track a) and *Hpa* I-cleaved *Bgl* II fragment B (0.03 mM, track b) were subjected to electrophoresis on a 2% polyacrylamide–0.5% agarose gel.

lymerase in vitro, the promoters for T7 species IIIb and VI (2). When R8 DNA is truncated by exonuclease III-S1 digestion to remove the class III promoter which controls T7 RNA species VI, a minor promoter for T7-specific RNA polymerase is activated whose RNA corresponds in molecular weight to the T3 RNA species IV promoter.

A more distant possibility is that this phenomenon is related to the intradomain transfer model of Von Hippel et al. (39), where DNA-

FIG. 8. Hybridization of isolated in vitro class II transcripts to *Hpa* I restriction fragments of *Bgl* II fragment B. Transfer of the *Hpa* I restriction fragments to nitrocellulose filter paper, isolation of separated 32 P-labeled transcripts, and hybridization were described in the text. Track (a) shows a photograph of the ethidium bromide-stained *Hpa* I fragments of *Bgl* II fragment B as observed in the agarose gel before their transfer to the nitrocellulose filter. Track (b) shows the resulting autoradiogram from the hybridization of transcript Y (140,000 cpm or 280 pmol of RNA) from *Hpa* I-cleaved *Bgl* II fragment B (see Fig. 7 and text).

TABLE 3. *Molecular weights expected for in vitro class II transcripts read from intact T7 DNA by T7-specific RNA polymerase*

Class II RNA species	Approximate promoter location (T7 units)	Mol wt ^a
VII	13.5	6.5×10^6
VIII	18.9	5.8×10^6
IX	22.6	5.3×10^6
X	27.9	4.6×10^6

^a Molecular weights are calculated for RNAs beginning at individual class II promoter sites (mapped as discussed in text) and continuing through to the strong terminator for T7-specific RNA polymerase at 61% on the T7 map.

binding proteins are hypothetically caged within a single DNA molecule. If the 14.6% promoter represents a binding site for T7-specific RNA polymerase that is found very quickly, but at which productive RNA chain initiation is slow for some reason, then another unoccupied promoter located on the same DNA molecule might be able to sequester this polymerase molecule by intrastrand transfer. This transfer would be blocked by cleavage of the DNA to isolate each promoter on a separate fragment. However, this model does not account for the stimulation of total template activity.

To analyze transcripts from class II promoters, we have had to cleave T7 DNA so that the class II promoters and class III promoters end up on separate DNA molecules. Because, as we discussed above, this cleavage may well affect their utilization by T7-specific RNA polymerase, it would be premature to make quantitative comparisons of the utilization of class II and class III promoters which are normally on the same DNA molecule. However, even at near-saturating DNA concentrations, the rate of RNA synthesis with T7 *Bgl* II fragment B is much lower than with intact T7 DNA, which suggests that the class II promoter sites are much weaker than the class III promoter sites under our reaction conditions. Recently, McAllister and Wu have shown in hybridization studies (18) that lowering the $MgCl_2$ concentration in the T7-specific RNA polymerase reaction solution from 20 to 5 mM increases the efficiency of class II transcription relative to class III. We have confirmed this result using *Bgl* II-cleaved T7 DNA as a template for T7-specific RNA polymerase. By comparing the relative molar amounts of RNA species VIII* and RNA species V, we could observe a two- to threefold stimulation of promoter VIII* utilization when the $MgCl_2$ concentration was lowered from 20 to 5 mM (data not shown).

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ADDENDUM IN PROOF

After this paper was submitted for publication, Joseph Coleman generously sent us a manuscript of his recent work (J. L. Oakley, R. E. Strothcamp, A. H. Sarris, and J. E. Coleman, *Biochemistry*, in press). These authors found that the T7 DNA restriction fragment *Hpa* II-140 consists of two different fragments of identical size. The *Hpa* II-140 fragment that maps directly to the left of fragment *Hpa* II-138 in the class II region of the T7 genome (*Hpa* II-140a) was found not to contain a promoter for T7-specific RNA polymerase. This result indicates that the promoter for T7-specific RNA polymerase observed by Oakley and Coleman (24) on fragment *Hpa* II-138 (mapping at 14.6% on the T7 genome) and the promoter that we observe for T7 RNA species VII* are identical and resolves the discrepancy between our results.

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