The *rIIA* Gene of Bacteriophage T4. II. Regulation of Its Messenger RNA Synthesis

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ABSTRACT

When the *rII* genes are first introduced into cells which had been previously infected by T4 phage deleted for these genes, the kinetics of synthesis of *rIIA* and *rIIB* RNA are rapid and identical. We show that this rapid synthesis depends on a functional *motA* gene for *rIIB*, but not for *rIIA*, RNA synthesis. By primer-extension mapping of T4 messenger RNA, we find three promoters close to the *rIIA* gene. One of them is an early promoter just before the *rIIA.1* gene; it is used under all conditions tested. Another is in the coding portion of the *rIIA.1* gene; it is weak, primarily because of a 19-bp spacing between the −10 and −35 elements, and its use is stimulated by T4 functions. The third is a *motA*-dependent (middle) promoter which has an unusual CCCGCTT box at −33. We present results which suggest that none of these promoters is likely to be the site at which the *motB* and *motC* gene products exercise their major influence on *rIIA* RNA synthesis.

ALTHOUGH the *rIIA* gene of bacteriophage T4 has played an important role in the history of genetics, its role in the T4 life cycle is still obscure [see the companion study (DAEGELEN and BRODY 1990); and SINGER, SHINEDLING and GOLD 1983)]. The control of biosynthesis of the *rIIA* gene product is also not fully understood. The *rIIA* gene is part of an early transcription unit (a transcription unit for which initiation of RNA synthesis requires no T4 proteins). Moreover, there is a ρ-sensitive termination site between this early promoter and the *rIIA* gene (SEDEROFF, BOLLE and EPSTEIN 1971; SEDEROFF *et al.* 1971; DAEGELEN and BRODY 1976; CARUSO *et al.* 1979; PULITZER, COPPO and CARUSO 1979; YOUNG AND CRONE MENARD 1980; DAEGELEN, D'AUBENTON-CARAFA and BRODY 1982; THERMES and BRODY 1984). This picture of *rIIA* as a "classic" delayed-early gene of T4 (BRODY, RABUSSAY and HALL 1983) is clearly insufficient to explain a number of experimental results.

When T4 development is allowed to take place for several minutes before the *rII* genes are introduced into infected cells by superinfection, the kinetics of *rIIA* RNA synthesis are not of the delayed-early type; rather, *rIIA* and *rIIB* RNA appear with the same rapid kinetics (DAEGELEN and BRODY 1976). This suggests that a promoter exists close to the *rIIA* gene, as close, in fact, as is the middle *rIIB* promoter to the *rIIB* gene. This latter promoter determines an RNA start only 122 nucleotides upstream of the ATG of *rIIB* (SCHMIDT *et al.* 1970; PRIBNOW *et al.* 1981; GUILD *et al.* 1988). Such an *rIIA* promoter was found by SELZER *et al.* 1978; 1981). They showed that a DNA fragment containing the beginning of the *rIIA* gene and extending partially into the upstream gene *60* contains a promoter which drives *rIIA* RNA synthesis when cloned into plasmid pBR313. The question remains why this promoter does not produce a detectable immediate-early transcript when it is part of an injected T4 DNA molecule.

The expression of *rIIA* RNA in the middle mode is also unclear. Elimination of the *motA* gene product, a positive activator of middle-mode transcription (BRODY, RABUSSAY and HALL 1983; GUILD *et al.* 1988), does not eliminate *rIIA* RNA synthesis (DAEGELEN, D'AUBENTON-CARAFA and BRODY 1982; PULITZER, COLOMBO and CIARAMELLA 1985). In fact, it leads to overproduction of the rIIA protein (MATTSON, RICHARDSON and GOODIN 1974; MATTSON, VAN HOUWE and EPSTEIN 1978). However, simultaneously eliminating *motA* function and deleting two other loci of T4 (*motB* and *motC*) does lead to a reduction of *rIIA* RNA synthesis (PULITZER, COLOMBO and CIARAMELLA 1985). The roles of *motB* and *motC* are not clear, although the motB protein has been identified and has been shown to be localized in the same prereplicative DNA-protein complex as the motA protein (UZAN *et al.* 1985) . Finally, *rIIA* RNA is known to be translationally repressed by the T4 regA protein (WIBERG and KARAM 1983; MILLER *et al.* 1987; WINTER *et al.* 1987; also, see DAEGELEN and BRODY 1990).

Armed with the DNA sequence of the *rIIA* gene, we have reinvestigated *rIIA* expression. We have iden-

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TABLE 1

T4 bacteriophage strains used in these studies

Strain	Gene	Origin
B+		Our collection
D+		Our collection
AP80	*rIIA*[a]	R. H. EPSTEIN
AP129	*rIIA*[a]	R. H. EPSTEIN
F120	*rIIA*[a]	R. H. EPSTEIN
744	*rIIA*[a]	R. H. EPSTEIN
859	*rIIA*[a]	R. H. EPSTEIN
H88	*del(rIIA)*[a]	R. H. EPSTEIN
638	*del(rIIB)*[a]	R. H. EPSTEIN
tsG1	*motA*[b]	T. MATTSON
amG1	*motA*[c]	T. MATTSON
amN122	*42*	Our collection
amE645 amG1	*42, motA*	Our collection
del(39-56)-1	Many genes[d]	J. F. PULITZER[f]
del(39-56)-3	Many genes[d]	J. F. PULITZER[f]
del(39-56)-4	Many genes[d]	J. F. PULITZER[f]
del(39-56)-1 amG1	Many genes, *motA*	J. F. PULITZER[f]
del(39-56)-3 amG1	Many genes, *motA*	J. F. PULITZER[f]
del(39-56)-4 amG1	Many genes, *motA*	J. F. PULTIZER[f]
del(39-56)-11 amG1	Many genes[d], *motA*	J. F. PULITZER[f]
del(39-56)-1 amG1 comC55-6	Many genes, *motA, comC-α*[e]	J. F. PULITZER[f]
del(39-56)-3 amG1 comC55-6	Many genes, *motA, comC-α*	J. F. PULITZER[f]
isar: *imm2*$^-$ *s amNG205 r1589*	*imm, s, 42, del(rIIA-rIIB)*	Our collection[g]
tisar: isar *tsG1*	*imm, s, 42, del(rIIA-rIIB), motA*	T. MATTSON[h]

[a] BENZER (1961).
[b] MATTSON, RICHARDSON and GOODIN (1974).
[c] MATTSON, VAN HOUWE and EPSTEIN (1978).
[d] HOMYK and WEIL (1974). The *del(39-56)* mutants contain overlapping deletions covering about 10 kbp between genes 39 and 56. The original *del(39-56)-1*, -3, -4 and -11 contained the *rII* deletion *r1589*.
[e] CARUSO *et al.* (1979).
[f] We thank JOHN F. PULITZER for giving us the *rII*$^+$ single, double and triple mutant *del(39-56)* strains.
[g] DAEGELEN and BRODY (1976).
[h] We thank TOM MATTSON for the construction of the T4*isar* phage starting with T4*isar* and *tsG1* phages.

tified three promoters just upstream of the *rIIA* gene and have studied the control of their utilization.

MATERIALS AND METHODS

Enzymes and biochemicals: Restriction endonucleases were purchased from New England Biolabs and Bethesda Research Laboratories (BRL). T4 DNA ligase and *Escherichia coli* DNA polymerase I Klenow fragment were purchased from Amersham or BRL. Avian myeloblastosis virus (AMV) reverse transcriptase came from Genofit (Geneva). T4 polynucleotide kinase, deoxy- and dideoxynucleotides, 17-mer M13 primer, deoxyadenosine 5′-[α-^{35}S]thiotriphosphate ([α^{35}]S-dATP), adenosine 5′-[γ-^{32}P]triphosphate ([γ^{32}P]ATP) and L-[^{35}S]methionine were all obtained from Amersham.

Plasmids: DNA of plasmid pTB101 was a gift from R. H. EPSTEIN (Geneva). Plasmid pTB101 is a derivative of pBR313 (BOLIVAR *et al.* 1977); it contains a 2-kbp *Eco*RI-*Hin*dIII fragment of T4 DNA. This fragment, which we shall call *60-A*, includes the distal portion of gene *60* and the proximal two-thirds of gene *rIIA* (SELZER *et al.* 1978, 1981). We shall use the name p60A for this plasmid.

Bacteriophages: All T4 strains used are described in Table 1. Phage M13mp18 (NORRANDER, KEMPE and MESSING 1983) was obtained from Amersham.

Bacteria: The p60A plasmid (SELZER*et al.* 1978, 1981) was transferred into the *E. coli* strain MC1061 (*F*$^-$ *araO*$_{139}$ *del(araABOIC-leu)*$_{7679}$ *del(lac)*$_{x74}$ *galK rpsL hsr*$^-$ *hsm*$^+$; CASADABAN and COHEN 1980). *E. coli* JM101 and JM105 strains obtained from Amersham were used as recipients for M13 phage DNA transformations and as hosts for the propagation of M13 phages. *E. coli* B^E (*su*$^-$) was the host for the growth of T4B and T4D phages.

T4 protein labeling and analysis: *E. coli* B^E was grown in M9 medium supplemented with 1% casamino acids at 30° (DAEGELEN and BRODY 1976). When bacteria reached 5×10^8 cells per ml, they were chilled on ice, then irradiated in 100-mm Petri dishes (provided with sterile magnetic stirrers; no more than 30 ml) for 8 min, 7.5 cm from a mineralight UVS12 lamp. After irradiation, the cells were kept on ice in the dark to minimize photoreactivation until infection could be carried out. Then, cells were preincubated 8 min at 42°, L-tryptophan was added to 25 μg/ml and, 1 min later, the primary phages were added at a multiplicity of infection (m.o.i.) of 2 (zero-time of the experiment). At 3 min the superinfecting phages were added at a m.o.i. of 5. Samples (500 μl) were pulse-labeled during 30 sec with 20 μCi of high specific activity L-[^{35}S]methionine. Radioactive labeling was stopped and phage development was arrested with the addition of 500 μl of ice cold 10% trichloracetic acid containing 100 μg/ml of L-methionine, followed by immediate chilling on ice. Labeled cells were kept at 0° overnight in Eppendorf tubes. Cell precipitates were centrifuged 15 min at 4°, then resuspended and washed with 1 ml of acetone. The suspension was left 2 hr

at −20°, then centrifuged as before. Acetone was evaporated at 37° for 30 min, and the pellet was resuspended in 100 μl of sample buffer (O'FARRELL, GOLD and HUANG 1973) and heated in a boiling water bath for 2 min. The proteins were analyzed by electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (1 mm thick, 17.5 cm long) composed of a discontinuous gradient of 5, 10, 12.5, 15 and 17.5 acrylamide, according to CARDILLO, LANDRY and WIBERG (1979). After staining, destaining and drying, the gels were subjected to autoradiography with Kodak X-Omat 5 high-speed X-ray films.

Hybridization to plasmid DNA: These experiments were carried out as described in DAEGELEN, D'AUBENTON-CARAFA and BRODY (1982).

T4 phage infection, T4 RNA purification, DNA sequencing, primer-extension reactions and *in vitro* synthesis of T4 RNA: These techniques are described in the MATERIALS AND METHODS section of our companion study (DAEGELEN and BRODY (1990).

RESULTS

Superinfection experiments: We have shown that *rIIA* and *rIIB* RNA appear with the same rapid kinetics when the *rII* genes are introduced into cells previously infected by phage deleted for these two genes (DAEGELEN and BRODY 1976). This experiment was possible to do because we had constructed a phage strain for primary infection, T4isar, which carried the *rIIA* and *B* deletion *NB2226*, mutations in the *imm* and *s* genes which permitted efficient superinfection, and a mutation in gene *42* which eliminated DNA synthesis. We ask here whether the rapid kinetics of *rIIA* (and *rIIB*) RNA synthesis seen when these genes are introduced by superinfection depend on the *motA* function in the primary phage T4isar. The mutant strain T4tisar (see Table 1) contains the mutations listed above plus the *motA tsG1* mutation the We start by examining the kinetics of protein synthesis in such superinfection experiments. Figure 1 shows these kinetics for three different conditions of infection. Panel 1-D shows a control experiment in which T4*amN122* (*42*$^-$) infects *E. coli* B^E at 42°. The rIIA protein is first seen in the pulse labeling from 1.75 to 2.25 min after infection. The maximal rate of rIIA protein synthesis is seen between 2.75 and 3.25 min after infection. The second series of pulse labelings (panels 1-A, 2-A and 3-A) are done using T4isar (*motA*$^+$) for a primary infection, superinfecting with T4*amN122* (*42*$^-$ *motA*$^+$) 3 min after primary infection, and pulse labeling afterward using the same protocol as was used for the simple infection with T4*amN122*. The rIIA protein was first detected between 4.25 and 4.75 min after infection with the primary phage, which is 1.25 to 1.75 min after introduction of the *rII* genes. The maximal rate is attained 2.5 to 3 min after superinfection (lane g). Thus, previously infected cells which are *motA*$^+$ express rIIA protein slightly faster (by about 30 sec) than do *E. coli* upon simple infection. This would correspond to an acceleration of about 1 min at 30°. The third series of pulse labelings (panels 1-B, 2-B and 3-B) are like those of section A except that both the primary infecting (T4tisar) and the superinfecting (T4*amE645 amG1: 42*$^-$ *motA*) phages are mutated in the *motA* gene. It is evident from the kinetics of protein synthesis that the rIIA protein still appears 1.25 to 1.75 min after superinfection. Thus, the early appearance of rIIA protein seems to be independent of *motA* function in these superinfection experiments. It is evident that the overproduction of rIIA protein seen in many *motA*$^-$ infections is also seen in these superinfection experiments (Figure 1; panels 2-B, lanes g to j).

The analysis of the rIIB protein in these experiments is complicated by the close proximity of another protein (probably gene *32* protein) on the SDS polyacrylamide gels. Nonetheless, it is clear that the production of rIIB protein introduced by superinfection, unlike rIIA protein, diminishes greatly when primary and superinfecting phages are *motA*$^-$.

We also show the kinetics of motA protein synthesis (Figure 1; panels 3-A and 3-B). Both the active motA protein (panel 3-A) and the inactive motA protein (panel 3-B) are synthesized up to 6–7 min at 42°. motA protein synthesis after simple infection at 42° is inhibited after 4 min (data not shown). We presume that this prolonged period of motA protein synthesis is derived from the *motA* gene of the superinfecting phage, but we do not know what mechanism is involved.

We have pulse-labeled RNA after superinfection to examine the sensitivity of *rIIA* and *rIIB* RNA synthesis to *motA* function in these experiments. The protocol is similar to that used for protein labeling: initial infection either with T4isar (*motA*$^+$) or T4tisar (*motA*$^-$) at 42°, a period of 3 min of early development, superinfection with either T4*amN122* (*42*$^-$ *motA*$^+$) for the *motA*$^+$ cells, or T4*amE645 amG1* (*42*$^-$ *motA*$^-$) for the *motA*$^-$ cells, and pulse labeling with [^{3}H]uridine at overlapping 30-sec intervals. [^{3}H]RNA samples were then hybridized to cloned fragments of genes *39*, *rIIA* or *rIIB* to determine synthesis rates. Gene *39* RNA (which is of course synthesized before superinfection) is synthesized at an identical rate whether or not *motA* is functional in the superinfection experiments. This is shown in Figure 2a and serves as a control for the *rII* analyses. In Figure 2b, we show the kinetics of appearance of *rIIB*-specific RNA (the probe being from the middle of the *rIIB* gene). When *motA* is functional, *rIIB* RNA synthesis after superinfection reaches a maximal rate by 1 min (at 42°) after the *rIIB* gene is introduced into the cell. In the absence of *motA* function, however, virtually no *rIIB* RNA is made until about 2 min after superinfection. Thus, the rapid appearance of *rIIB* RNA upon superinfection depends upon *motA* gene product, presumably

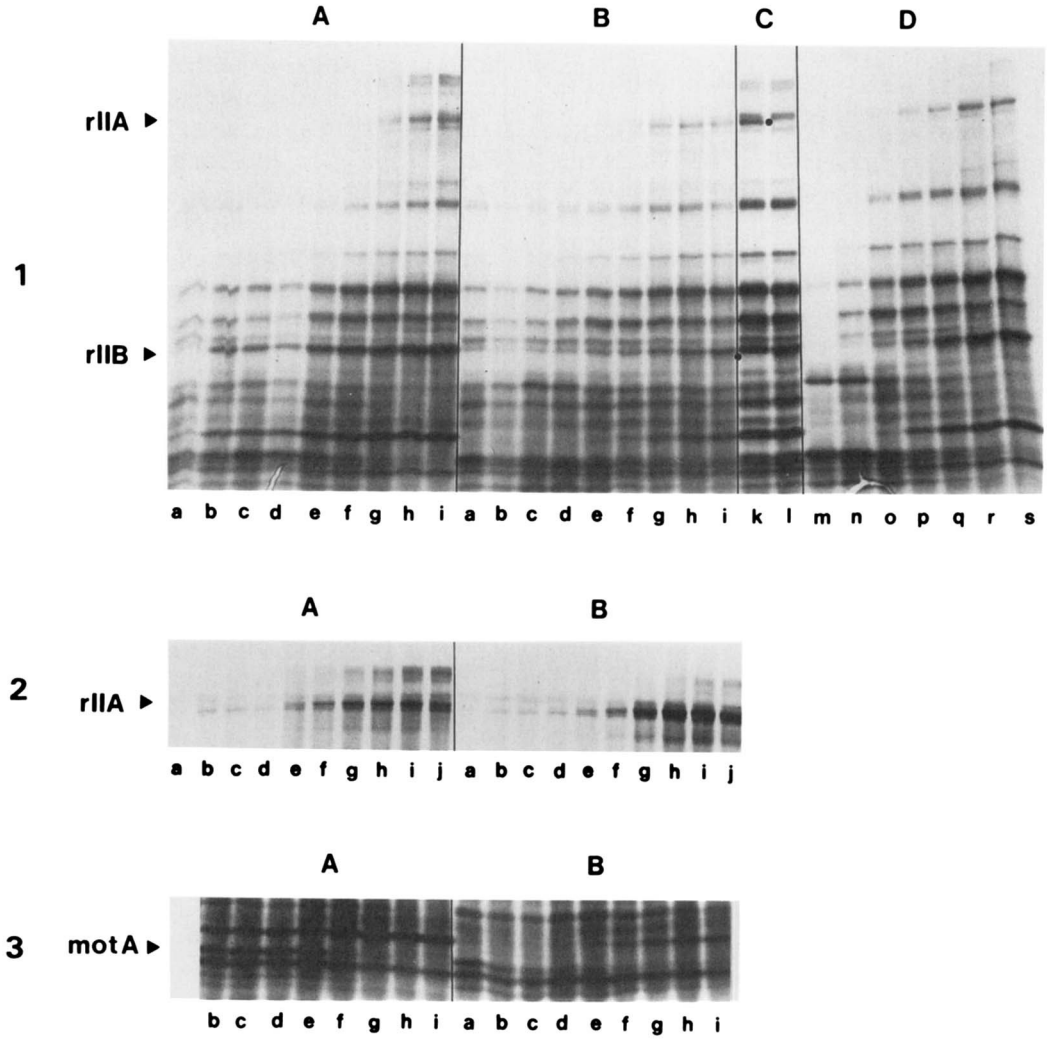

FIGURE 1.— SDS-polyacrylamide gel analysis in superinfection experiments. Panels 1-A and 1-B: Kinetics of *rIIA* and *rIIB* protein synthesis. *E. coli* B^E were grown in M9S at 37° to 5×10^8 cells/ml, chilled rapidly, UV-irradiated and then incubated in the dark for 8 min at 42°. Cells were then treated with L-tryptophan at 20 μg/ml and, 1 min later, infected at a m.o.i. of 2 with either T4isar (panel 1-A) or T4tisar (panel 1-B); this was the zero-time of infection. Three minutes after the primary infection, the cells were superinfected at a m.o.i. of 5 with either T4*amN122* (*42*$^-$; panel 1-A) or T4*amE645 amG1* (*42*$^-$ *motA*$^-$; panel 1-B). At different times before and after superinfection, aliquots of superinfected cells were labeled with L-[^{35}S]methionine (40 μCi/ml of infected culture; 1000 Ci/mmol) and the pulse labeling was teminated 30 sec later by addition of equal volumes of ice-cold 10% trichloracetic acid (TCA) containing 100 μg/ml of L-methionine and immediate chilling on ice. The proteins were then prepared and analyzed on SDS-polyacrylamide step-gradient gels (see MATERIALS AND METHODS for all experimental details). Each lane represents 500,000 cpm of TCA-precipitable material. From left to right: (a) 2.25 to 2.75 min; (b) 3.25 to 3.75 min; (c) 3.5 to 4 min; (d) 3.75 to 4.25 min; (e) 4.25 to 4.75 min; (f) 4.75 to 5.25 min; (g) 5.5 to 6 min; (h) 6.25 to 6.75 min; (i) 7.25 to7.75 min. Panels 2-A and 2-B: Details of the kinetics of rIIA protein synthesis. This is a part of an SDS-polyacrylamide gel similar to the one above. Experimental conditions were the same as in panels 1-A and 1-B, except for additional pulse-labeling (j) between 8.5 and 9 min. Panels 3-A and 3-B: Details of the kinetics of motA protein synthesis. Same as 2-A and 2-B. Panel 1-C: rIIA and rIIB protein synthesis in T4 *rII* deletions. *E. coli* B^E grown in M9S at 43° to 5×10^8 cells/ml were infected at a m.o.i. of 7 with either T4*638* (*delrIIB*; lane k) or T4*H88* (*delrIIA*; lane *1*). The cells were pulse-labeled with L-[^{35}S]methionine between 1 and 2 min after infection and treated as before. Each lane represents 500,000 cpm of TCA-precipitable material. The black dots mark the positions of rIIA and rIIB proteins on these SDS-polyacrylamide gels. Panel 1-D: Kinetics of rIIA and rIIB protein synthesis in a simple infection. *E. coli* B^E grown in M9S at 43° to 5×10^8 cells/ml were infected at a m.o.i. of 7 with T4*amN122* (*42*$^-$) Samples of the infected cells were pulse-labeled with L-[^{35}S]methionine and treated as above. From left to right: (m) 0.25 to 0.75 sec labeling time; (n) 0.75 to 1.25; (o) 1.25 to 1.75 min; (p) 1.75 to 2.25 min; (q) 2.25 to 2.75 min; (r) 2.75 to 3.25 min; (s) 3.25 to 3.75 min. Each lane represents 500,0000 cpm of TCA-precipitable material.

from that already accumulated after infection by T4isar (see above).

The situation for *rIIA* (Figure 2c) is entirely different. Although the kinetics of appearance of *rIIA* RNA are rapid after superinfection of *motA*$^+$ cells, the rapidity is not diminished by the elimination of *motA* function in the primary infection. Thus, the rapid appearance of *rIIA* and *rIIB* RNA seen in superinfected cells seem to be generated via different transcriptional pathways.

Primer extension analysis: Previously, we had postulated the existence of a promoter close to the *rIIA*

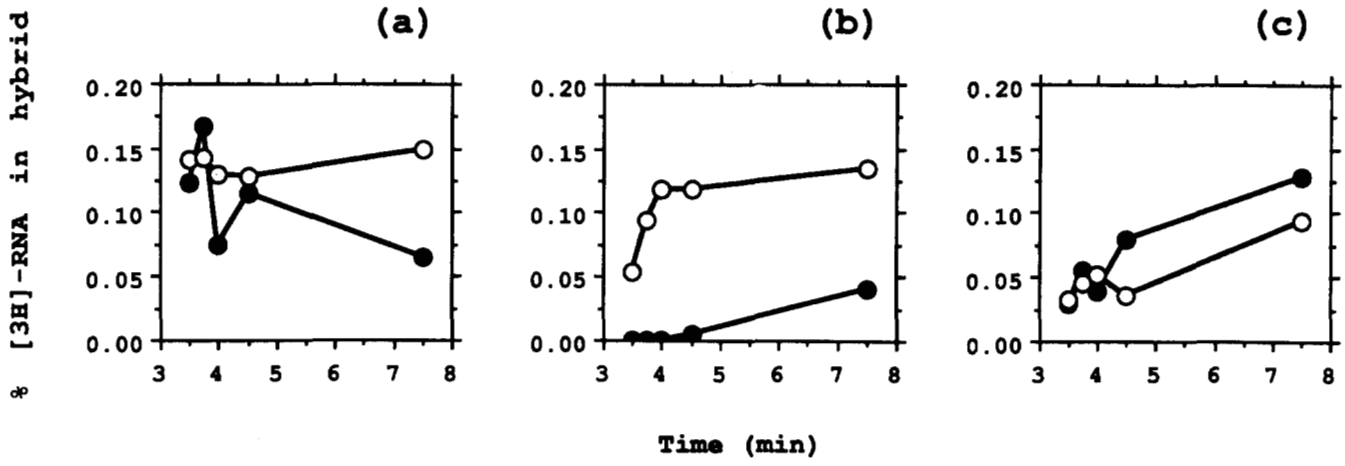

FIGURE 2.— Kinetics of gene *rIIA*, gene *rIIB* and gene *39* RNA synthesis in superinfection experiments. *E. coli* B^E cells were grown in M9S at 42° to 5 × 10^8 cells/ml and treated with L-tryptophan at 20 μg/ml; 1 min later, they were infected with the primary phage, either T4isar or T4tisar, at a m.o.i. of 2; this is the zero time for the experiment. Three minutes later the superinfecting phage were added at a m.o.i. of 5, either with T4*amN122* (*42*$^-$) or T4*amE645 amG1* (*42*$^-$ *motA*$^-$). At different times after superinfection, 500-μl samples of T4-infected cells were transferred to flasks prewarmed at 42° and containing 100 μCi of [5-^{3}H]uridine (29.4 Ci/mmol) . Pulse-labeling was terminated 30 sec later by adding ice and 500 μl of an ice-cold solution of unlabeled uridine at 1 mg/ml. After centrifugation, ^{3}H-labeled RNA was extracted, then analyzed by hybridization to plasmids containing T4 DNA inserts (see MATERIALS AND METHODS for details). The plasmids used were p626 (2565 bp covering gene *39*), p615 (109 bp internal to gene *rIIB*) and p60A (1970 bp covering the distal part of gene *60*, ORF *rIIA.1* and the proximal two-thirds part of gene *rIIA*). Hybridization to plasmid pBR322 (the cloning vehicle for p626 and p615; BOLIVAR *et al.* 1977) was done in parallel as a control experiment to obtain the background level for each hybridization experiment. We assumed that using pBR322 in place of pBR313 (the cloning vehicle for the *60-rIIA* T4 DNA fragment) makes no difference in establishing the background level of *rIIA* RNA determination. 1) Pulse-labeling from 3.25 to 3.75 min; isar: 164,160 cpm TCA-precipitable, 17 cpm hybridized to pBR322, 738 cpm hybridized to p626, 32 cpm hybridized to p615, 180 cpm hybridized to p60A; tisar: 54,045 cpm TCA-precipitable, 9 cpm hybridized to pBR322, 182 cpm hybridized to p626, 11 cpm hybridized to p615, 56 cpm hybridized to p60A. 2) 3.5 to 4 min; isar: 215,325, 18, 809, 47, 287; tisar: 59,220, 9, 308, 12, 91. 3) 3.75 to 4.25 min; isar: 271,710, 20, 926, 64, 391; tisar: 75,780, 10, 158, 13, 89. 4) 4.25 to 4.75 min; isar: 324,180, 24, 1,090, 74, 354; tisar: 43,650, 9, 139, 12, 90. 5) 7.25 min; isar: 109,755, 12, 433, 31, 251; tisar: 30,555, 9, 61, 12, 94. The plasmid hybridizations are expressed as the percentage of ^{3}H cpm annealed to each DNA plasmid (minus the cpm annealed to pBR322 plasmid DNA) in the TCA-precipitable ^{3}H cpm for 1000 bp of T4 DNA insert. (a) p626, gene *39*; (b) p615, gene *rIIB*; (c) p60A, gene *rIIA*. The isar results are shown as open circles and the tisar results as filled circles.

gene (DAEGELEN and BRODY 1976). SELZER *et al.* 1978, 1981) found such a promoter in the *60-rIIA* fragment, active when cloned into pBR313. Above, we have presented experiments that suggest that such a promoter will not be *motA*-dependent (BRODY, RABUSSAY and HALL 1983; GUILD *et al.* 1988) We have used primer extension analysis to map 5′ ends of RNA in and around the beginning of the *rIIA* gene. Using oligonucleotide R2A2 (see Table 2) as a primer for AMV reverse transcriptase, we have analyzed a large collection of RNA preparations.

First of all, RNA extracted from uninfected *E. coli* containing the p60A plasmid possesses one 5′ end when probed with r2A2 (Figure 3). It maps to the G at position 313 of our sequence, just before the ORF *rIIA.1* (see Figure 2 of DAEGELEN and BRODY 1990). This 5′ end implies a −10 sequence of TATAAT, and a −35 sequence of TTTACT, with a 17-bp spacer; these three elements are frequently found in the T4 genome as early promoters. In fact, this *60-rIIA* promoter has been cloned as a T4 early promoter in the collection of LIEBIG and RÜGER (1989). It corresponds to their p 1,4–3,9. It has a score of 72.2 in the promoter search program of MULLIGAN *et al.* (1984), which is equal to the strengths of the T7 promoters. Thus, the existence of this promoter in p60A explains both the results of SELZER *et al.* (1978, 1981) and our superinfection results. What of course it does not explain is why *rIIA* is not an immediate-early gene (DAEGELEN and BRODY 1976; SEDEROFF, BOLLE and EPSTEIN 1971; SEDEROFF *et al.* 1971; DAEGELEN, D'AUBENTON-CARAFA and BRODY 1982). We shall call this promoter P1.

We then analyzed RNA extracted 3, 7, 10 and 15 min after infection at 30° with T4B$^+$, T4*del* (*39–56*)-*11* (*motB*$^-$ *motC*$^-$) or T4*amG1* (*motA*$^-$) at 30° (Figure 3). In all of these RNA preparations, the early *rIIA* P1 promoter identified in p60A RNA is used. The *rIIA* RNA seems to be synthesized primarily prereplicatively; the 15-min RNA in all infections contain less *rIIA* RNA than the 7-min RNA. There are two other 5′ ends generated after T4*B*$^+$ and T4*del*(*39–56*)-*11* infections and one other generated after T4*amG1* (*motA*$^-$) infection. The *motA*-dependent 5′ end maps to the middle of the ORF *rIIA.1*. The +1 is a doublet, the C and G at nucleotides 473 and 474, respectively, of our sequence (see Figure 2 of DAEGELEN and BRODY 1990). They imply a −10 of

TABLE 2

T4-specific oligodeoxynucleotides

Name	Length	Sequence	Location[a]
r2A1	20-mer	5′-GATAACTTGATGCACGGCTG-3′	1703-1723
r2A2	19-mer	5′-GAACCATTACCAAGAATTG-3′	572–590
r2A3	23-mer	5′-CATTAAGTGCATGAGCATCAATC-3′	702–724
r2A5	23-mer	5′-CGTTTAGCTGAACTTGAATAACC-3′	2362–2384
r2B1	20-mer	5′-CAGCCAATTCCTGTTGGGTG-3′	2807–2826

[a] The location refers to the sequence shown in Figure 2 of our companion study [DAEGELEN and BRODY (1990)].

TAAAAT and a −33 of CCCGCTT. This is, by its *motA*$^-$ sensitivity, a middle promoter. It is, nonetheless, unique. (One CCCGCTT motif is found in our 75-kbp T4 DNA sequence bank, 75 bp after the ATG initiator of gene *44*. GUILD *et al.* (1988) have mapped 14 *motA*-dependent promoters and have found a consensus sequence at −33 of (AT) (AT)TGCTT. The *rIIA* middle promoter has the GCTT which is conserved in all middle promoters but this sequence is preceded by three C · G base pairs. We note as well that the −33 CCCGCTT is surrounded on either side by a TTTAAA palindrome. We thought that such a constellation of sequences might reflect binding sites for both motA (the CCCGCTT) and motB (TTTAAA) proteins. The *motB* locus, as we shall show, does not seem to effect recognition of this *motA*-dependent promoter. This is clearly demonstrated in Figure 3, where T4*del(39–56)-11* (*motB*$^-$ *motC*$^-$) RNA is shown to have 5′ ends at 473 and 474, which are indistinguishable from those of wild-type RNA. Thus, the *rIIA* middle promoter, despite its unusual sequence, seems to require only motA protein function for its utilization. We shall call this promoter PM.

There is a third 5′ end which maps between the two described above, also in the coding region of ORF *rIIA.1*. This 5′ end maps to the T, at 405 in the sequence given in Figure 2 of DAEGELEN and BRODY (1990). Examining the DNA sequence upstream of this 5′ end, we find a −10 TAGAAT sequence 1 bp different from the *E. coli* −10 consensus sequence, and a −35 TTGATA box again 1 bp different from the *E. coli* consensus sequence. These two elements are separated by 19 bp, which is not optimal for promoter function. Promoters are known, nonetheless, which have −10 and −35 elements separated by 19 bp. The score of this putative promoter in the test of MULLIGAN *et al.* (1984) is 52.7, about the strength of the pBR322 *bla* promoter. Is this 5′ end generated by initiation of RNA synthesis? If it were not, one might imagine that it is an artefact of primer extension, because very strong secondary structure can cause the AMV reverse transcriptase to terminate (TUERK *et al.* 1988) . Alternatively, it could be a processing site, where RNA generated from upstream promoters is cleaved. We now argue that it is neither of these, but is in fact a 5′ end generated by the promoter described above. Examination of the sequence around this 5′ end shows no evidence of strong secondary structure. Moreover, RNA structure would cause the reverse transcriptase to stop independently of the source of the RNA. The RNA starting at this 5′ end, which we will call P2 RNA, is completely absent when *rIIA* RNA is generated from the p60A plasmid; only P1 is used. This is true even when the autoradiographs are heavily overexposed (data not shown). Therefore, reverse transcriptase can pass through this region without stopping. This conclusion is reinforced by the weak P2 band (compared to the P1 band) seen after infection with T4 phage carrying deletions between genes *39* and *56*, as we shall discuss below. Could band 2 RNA arise, then, by processing? It could not, because RNA synthesized *in vitro* also shows the 5′ end of P2 RNA (Figure 4). We conclude, then, that this 5′ end is generated from the P2 promoter. When we compare *in vitro* RNA with 5-min *in vivo* RNA, we see a striking result. P1-generated transcripts predominate in *in vitro* RNA; P2 use is weak, and the middle promoter is not used at all. *In vivo*, P1 is used as it is *in vitro*, the middle promoter PM is now used in a *motA*-dependent manner (as we shall show below), and P2 use is greatly stimulated. In fact, P2 use depends on T4 protein synthesis almost as much as PM utilization depends on *motA* function. We shall come back to this observation further on.

When T4 infects *E. coli* in the presence of the antibiotic chloramphenicol (CAM), no T4 proteins are made, and one would thus expect a pattern approximating *in vitro* RNA. This, in fact, is what one sees, although P2 RNA is visible only on long exposures of the polyacrylamide gel presented in Figure 4. What is most remarkable in the T4 CAM RNA isolated 5 min after infection at 30° is the low level of signal, both P1 and P2. We think this is probably due to intragenic polarity, in that the RNA generated from the P1 and P2 promoters is degraded rapidly, so that by 5 min after infection, very little RNA is left to hybridize to the R2A2 probe. We have previously documented such intragenic polarity in transcription of the *motA* gene (UZAN *et al.* 1988). There is, none-

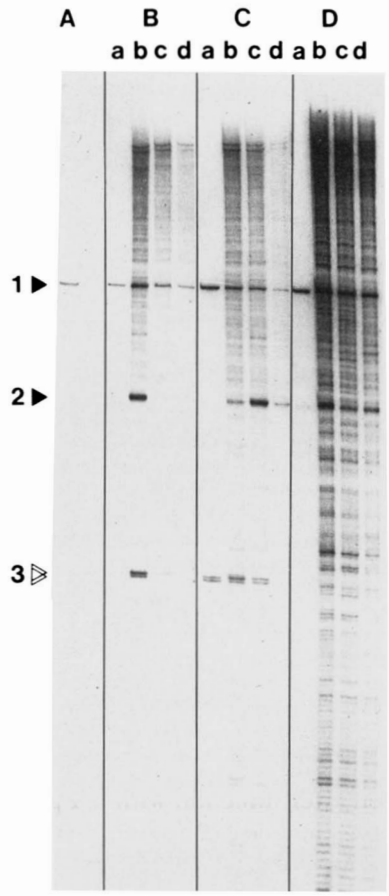

FIGURE 3.— Mapping of the 5′ ends of *rIIA.1* and *rIIA* RNA. The three 5′ ends just upstream of gene *rIIA* were determined by primer-extension analysis using as templates the RNA preparation from various sources, the r2A2 oligodeoxynucleotide primer, and AMV reverse transcriptase. The labeled DNA products were analyzed on a denaturing 6% polyacrylamide gel. (A) Expression of the P1 promoter in the plasmid p60A. This lane shows a primer-extension experiment with the RNA prepared from *E. coli* strain MC1061 containing the 1970-bp T4 DNA fragment *60-rIIA* inserted into pBR313. Cells were grown overnight at 37°. The filled arrow 1 points to the 5′ end generated by the P1 promoter. (B) Kinetics of appearance of 5′ ends of *rIIA.1* and *rIIA* RNA in *E. coli* infected with T4B$^+$ wild type. *E. coli* B^F were grown in M9S at 30° to 5 × 10^8 cells/ml, treated with L-tryptophan at 20 μg/ml and infected, 1 min later, with T4B$^+$ phage at a m.o.i. of 5. At various times after infection, samples were pipetted into lysis buffer (see MATERIALS AND METHODS). After lysis, each RNA preparation was extracted and used as a template for primer-extension. Lane (a): RNA extracted from cells after 3 min of infection; (b) 7 min; (c) 10 min; (d) 15 min. Filled arrows 1 and 2 point to P1 and P2 promoters, respectively. Open arrows 3 point to the two 5′ ends corresponding to the PM promoter. (C) and (D) represent experiments similar to those in (B) except that the phages were T4*del(39–56)-11* and T4*amG1*(*motA*$^-$), respectively.

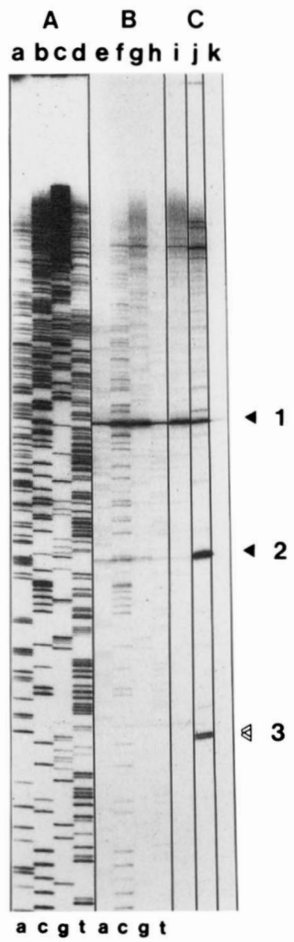

FIGURE 4.— *In vivo* and *in vitro* expression of *rIIA* promoters analyzed by primer-extension with the r2A2 oligodeoxynucleotide primer. From left to right: (A) Nucleotide sequence of gene *rIIA*. Single-stranded DNA purified from the 437-bp *Alu*I-*Acc*I T4 DNA fragment cloned into M13mp18 (see DAEGELEN and BRODY 1990) was sequenced by the SANGER dideoxynucleotide chain-termination method using the Klenow DNA polymerase (SANGER, NICKLEN and COULSON 1977). The products of the reaction were analyzed on a denaturing 6% polyacrylamide gel (lanes a, b, c and d). (B). A T4 RNA template synthesized *in vitro* with T4D$^+$ DNA and *E. coli* RNA polymerase (see MATERIALS AND METHODS for details) was reverse-transcribed by AMV reverse transcriptase. Each lane (e, f, g and h) corresponds to a standard dideoxynucleotide sequencing reaction. (C) Mapping of the *rIIA* promoters by primer-extension analysis of: (i) *in vitro* T4D$^+$ RNA; (j) *in vivo* T4D$^+$ RNA isolated 5 min after T4D$^+$ infection of *E. coli* cells at 30°; (k) *in vivo* T4D$^+$ RNA isolated 5 min after T4D$^+$ infection of *E. coli* cells that had been treated with 200 μg/ml of chloramphenicol 5 min before infection. The arrows 1, 2 and 3 point to P1, P2 and PM promoters, respectively,

theless, another possible explanation which we shall present in the discussion of our data.

The amounts of P1, P2 and PM RNA at various times after infection with T4 wild type, T4*del(39–56)-11* and T4*amG1* are shown in Figure 3. It is also clear from Figure 3 that some *rIIA* RNA is synthesized starting at promoters upstream of P1. We have not quantified what percentage of *rIIA* RNA derives from such upstream promoters. The maximal amount of P1 RNA is seen at 7 min after infection for all three phage infections. PM RNA is also maximal at 7 min after infection for wild-type and T4*del(39–56)-11* infections. It is, of course, not seen at all after infection by T4*amG1*. Of interest are the kinetics of accumulation of P2 RNA after these infections. P2 RNA is maximal at 7 min after infection with T4 wild type

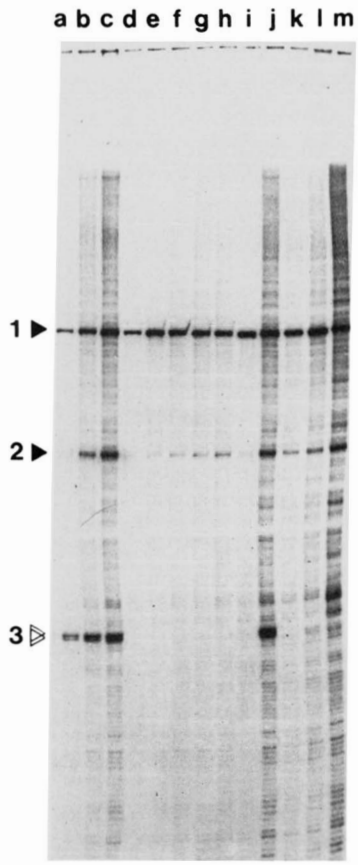

FIGURE 5.— Utilization of P1, P2 and PM *rIIA* promoters in various T4 mutant strains: primer-extension mapping of the 5′ ends generated by the promoters of *rIIA*. All experimental conditions were the same as in part (B) of Figure 3 except that RNA was prepared from *E. coli* B^F cells infected with various T4 mutant strains for 5 min at 30° for the samples shown in lanes (a) to (l), and for 3 min at 42° for lane (m). From left to right: (a) RNA prepared after infection with T4*del(39–56)-1*; (b) T4*del(39-56)-3*; (c) T4*del(39-56)-4*; (d) T4*del(39-56)-3 amG1*; (e) T4*del(39-56)4 amG1*; (f) T4*del(39-56)-1 amG1 comC55–6*; (g) T4*del(39-56)-3 amG1 comC55–6*; (h) T4*del(39-56)-1 amG1*; (i) T4*del(39-56)-11 amG1*; (j) $T4B^+$; (k) T4*amG1*; (l) T4*tsG1*; (m) T4*tsG1*.

and T4*amG1*, but the accumulation of P2 RNA is clearly retarded after infection with T4*del(39–56)*-11; maximal RNA levels are delayed until 10 min after infection. T4*del(39–56)-11* deletes, among others, genes *motB* and *motC*, which are thought to play a role in the rate of synthesis of *rIIA* RNA (PULITZER, COLOMBO and CIARAMELLA 1985). The *39–56* region is examined more closely in Figure 5 and Table 3. We have used several mutant strains to examine the role of *motB* and *motC* as well as the role of the point mutant, *comC55–6*, in the *comC-α* gene (TAKAHASHI 1978; CARUSO *et al.* 1979; PULITZER, COPPO and CARUSO 1979; J. F. PULITZER, personal communication) to see how utilization of these promoters correlates with studies of *rIIA* RNA synthesis measured by hybridization of [^{3}H]uridine pulse-labeled RNA to a small fragment of DNA from the interior of the *rIIA* gene (PULITZER, COLOMBO and CIARAMELLA 1985; J. F. PULITZER, personal communication). RNA isolated 5 min after infection at 30° (or 3 min after T4*tsG1* infection at 42°) with single, double and triple mutants are analyzed by primer extension. The P1 promoter is used to a similar degree in all these infections; no influence of the T4 regions tested here has ever been seen. PM acts like a simple *motA*-dependent promoter, no matter what other mutants we have combined with it. This is a first example of the lack of correlation of utilization of the *rIIA* promoters with the results of PULITZER *et al.* on RNA synthesis rates. We shall discuss this extensively later on. Here we simply note that the use of the *motA*-dependent promoter does not depend on *motB*, *motC* or *comC-α* gene products. The use of P2 is, as shown above, retarded in T4*del(39–56)-11*, which eliminates *motB* and *motC*. When T4*del(39–56)-11 amG1* (*motA*$^-$ *motB*$^-$ *motC*$^-$) infects *E. coli*, P2 use at 5 min is weak compared to $T4^+$ infection. The use of P2 is variably inhibited in the other deletions, but there is no suppressing effect of the *comC-α* mutation, *comC55–6*, for P2 utilization. This is important because PULITZER, COLOMBO and CIARAMELLA (1985) have found that this mutation suppresses the defect of *rIIA* RNA synthesis rates found in T4*del(39–56)-1 amG1* and T4*del(39–56)-3 amG1* infections (J. F. PULITZER, personal communication). Although it seems that *amG1* coupled with the deletions between genes *39* and *56* diminishes P2 utilization compared to the single mutants, there is no correlation with *motB* activity. In addition, we know

TABLE 3

Expression of P2 and PM promoters relative to P1

	Lane[a]												
	a	b	c	d	e	f	g	h	i	j	k	l	m
Gene	Genotype[b]												
motA	+	+	+	−	−	−	−	−	−	+	−	+−	−
motB	−	+	−	+	+	−	−	−	−	+	+	+	+
motC	+	−	−	−	−	+	−	+	−	+	+	+	+
comC-α	+	+	+	+	+	−[c]	−[c]	+	+	+	+	+	+
Promoter	Activity level[d]												
P2	20	31	42	6	7	11	14	20	7	55	21	26	47
PM	100	76	58	0	0	0	0	0	0	129	0	0	0

[a] Each lane from the gel used for the measure of P1, P2 and PM *rIIA* promoter utilization in various T4 mutant strains (see Figure 5, lanes a to m) has been scanned with a Vernon Phi 5 microdensitometer.

[b] A + or a − symbol corresponds to experimental conditions where the gene product is respectively active or inactive. The +− symbol means that the *tsG1* gene product is partially active at 30°.

[c] The *comC55-6* allele has a *comC-α*$^-$ phenotype at 30° (J. F. PULITZER, personal communication).

[d] The values represent, in arbitrary units, the integration of the density measured for the areas covering bands 1, 2 and 3. The areas for promoter P2 (band 2) and promoter PM (band 3) have been normalized relative to the areas found for promoter P1 (band 1 being given the value of 100) in each lane. No correction has been made for the minor bands representing background in each lane.

that this effect is only a delay in promoter utilization; slightly different development rates in the various infections may be misleading in comparing the relative use of P2.

Because use of P1, P2 and PM could not be simply correlated with the data on *rIIA* RNA synthesis rates, we probed the *rIIA* gene over its entire length (using r2A1, r2A2, r2A3 and r2A5 primers; see Table 2) to see whether *motB*, *motC* or the *comC-α* allele could influence the stability of *rIIA* RNA or the amount of *rIIA* found in these distal regions. We found no effect of the various alleles on the primer extension patterns in these distal regions (data not shown).

As a control for the experiments on *rIIA* RNA levels, we examined by primer extension the quantity of *rIIB* RNA found in the various mutant infections (using r2B1 primer; see Table 2). The results are shown in Figure 6 and shed some light on the *rIIA* data. The *rIIB* gene is transcribed in two different modes. There are two *motA*-dependent middle promoters for *rIIB* just upstream of the gene; they are, in fact, in the distal tip of the *rIIA* gene. In $motA^-$ infections, *rIIB* protein synthesis is severely delayed, although pulse labeling of *rIIB* RNA shows normal synthesis rates (PULITZER, COLOMBO and CIARAMELLA 1985). This is not surprising because *rIIB* RNA can also be synthesized from upstream early promoters, either the *rIIA* early promoters or early promoters further upstream. The delay in protein synthesis is thought to occur because these polycistronic *rIIB* RNA species are poorly translatable (MATTSON, VAN HOUWE and EPSTEIN 1978; DAEGELEN, D'AUBENTON-CARAFA and BRODY 1982; THERMES and BRODY 1984). The primer-extension data in Figure 6 demonstrate clearly the existence of the two middle promoters for *rIIB*, and show that they are *motA*-dependent. No other RNA 5′ ends are seen and no other alleles (among *motB*, *motC* and *comC-α*) influence the *rIIB* middle promoters. These data should be contrasted to Figure 7 of PULITZER, COLOMBO and CIRAMELLA (1985) where *rIIB* RNA synthesis rates have been measured by hybridizing pulse-labeled RNA to a fragment of *rIIB* DNA. $T4B^+$ and T4*amG1* have identical rates, whereas synthesis rates are depressed for $motA^-$ $motB^-$ and $motB^-$ $motC^-$ infections. We conclude from these experiments that the RNA from cells infected with T4*amG1* is either synthesized and turned over at equal rates or, more likely, that the lack of 5′ ends in the range of our polyacrylamide gel analyses simply gives a false notion of how much *rIIB* RNA is actually in the cell. The early *rIIB* promoters are too far upstream of the R2B1 probe to give a discernable signal in our primer-extension experiments. The implication of this result for the *rIIA* analyses is discussed below.

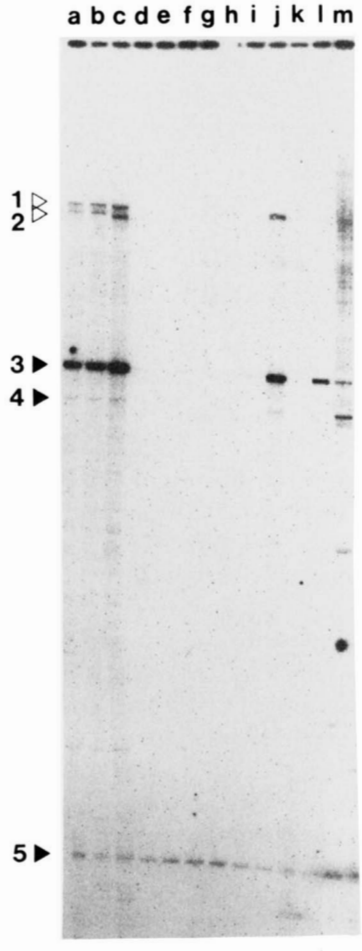

FIGURE 6.— -Utilization of *rIIB* promoters in various T4 mutant strains. The same RNA preparations used in Figure 5 to probe *rIIA* promoters have been used here to map the 5′ ends of RNA of gene *rIIB*. These RNA preparations have been analyzed by primer-extension using the r2B1 oligodeoxynucleotide primer. Open arrows 1 and 2 correspond to the P_{rIIB1} band in Figure 1 of GUILD *et al.* (1988). Filled arrow 3 points to the major *motA*-dependent *rIIB* proximal promoter, corresponding to the P_{rIIB2} band in Figure 1 of GUILD *et al.* (1988). Filled arrows 4 and 5 point to minor 5′ ends which we have not investigated further. From left to right: (a) RNA prepared after infection with T4*del(39–56)-1*; (b) T4*del(39–56)-3*; (c) T4*del(39–56)-4*; (d) T4*del(39–56)-3 amG1*; (e) T4*del(39–56)-4 amG1*; (f) T4*del(39–56)-1 amG1 comc55–6*; (g) T4*del(39–56)-3 amG1 comC55–6*; (h) T4*del(39–56)-1 amG1*; (i) T4*del(39–56)-11 amG1*; (j) $T4B^+$; (k) T4*amG1*; (l) T4*tsG1* at 30°; (m) T4*tsG1* at 42°.

DISCUSSION

Our data suggest that the rapid synthesis of *rIIA* RNA seen when T4 superinfects previously infected cells (DAEGELEN and BRODY 1976) takes place because T4 infection activates a promoter close to the *rIIA* gene. The superinfection experiments presented here with T4tisar indicate that it is not necessary to have an active *motA* gene for such activation to take place. This is in contrast to *rIIB* RNA synthesis, for which activation of the middle promoters close to the *rIIB* gene requires *motA* function. When we searched for promoters close to the *rIIA* gene, we found not one but three.

PM is a middle promoter for *rIIA* with a unique −33 box. The conserved GCTT is preceded by CCC whereas, in almost every other middle promoter, two A · T base pairs precede the conserved TGCTT (GUILD *et al.* 1988). These base pairs would not be dC · dG in mature T4 DNA, but rather dHmC · dGglucose; the glucosyl residues lie in the major groove and render this DNA segment relatively nondeformable compared to unglucosylated DNA. Nonetheless, we find that utilization of this promoter seems to depend on *motA* function in a simple way, just as do other, canonical middle promoters. The *motB*, *motC* and *comC-α* mutations tested here have no influence on the *motA* dependency of this middle *rIIA* promoter. Because *rIIA* RNA and protein synthesis do not depend on *motA* function, *rIIA* protein even being overexpressed in *motA*$^-$ conditions, we conclude that PM is a weak promoter relative to the other modes of expressing *rIIA* RNA. It is even possible that *motA* protein interaction with the unusual CCCGCTT box leads to a protein-DNA complex which weakly promotes transcription but dissociates poorly, thus acting as a block to elongation of *rIIA* RNA expressed via upstream promoters. This could explain the overproduction of *rIIA* protein in a *motA*$^-$ infection. It is, however, not the only possibility of *motA* repression of *rIIA* transcription, as we mention below.

P2 is a weak early promoter, used relatively inefficiently by unmodified *E. coli* RNA polymerase when it transcribes T4 DNA *in vitro*. This weakness is expected from the calculated score on the matrix of MULLIGAN *et al.* (1984). The poor theoretical score and, presumably, the low utilization *in vitro* are primarily a result of the 19-bp spacing between the −10 and −35 elements of P2. Use of this P2 promoter, as measured relative to the 5′ end generated by the P1 promoter, is stimulated by T4 infection. We have not localized precisely the elements in T4-infected cells that lead to this stimulation, but have found that a region of 10 kbp covered by the deletion *(39–56)-11* is necessary for the rapid stimulation of P2. This region covers the two loci, *motB* and *motC*, which can influence, under certain conditions, the rate of RNA synthesis of *rIIA* (PULITZER, COLOMBO and CIRAMRELLA 1985; J. F. PULITZER, personal communication). Because P2 stimulation occurs with a delay in T4*del(39–56)-11* infection, there must be still other T4 functions which can stimulate its use. We find no more than a small effect of *motA* on P2 use, although this effect is much larger when coupled with the effect of *motB* and *motC* (Figure 5). This is important because in the 19-bp spacer between the −35 and −10 elements of P2 is the sequence AAGCAGT which is the complement of ACTGCTT; in other words, there exists in this spacer region a potential motA protein binding site pointing in the sense opposed to early transcription. This would seem to be another possible way in which motA protein could repress *rIIA* RNA synthesis; our experiments do not detect such a repression.

The P1 promoter is used under all *in vivo* and *in vitro* conditions tested in our experiments. It is, by this definition a "classical" early promoter, requiring no T4 functions for its expression (BRODY, RABUSSAY and HALL 1983). It is, moreover, the only promoter used when the T4 genome segment *60-rIIA* is cloned into the plasmid *pBR313*, and it is responsible for *rIIA* expression in this plasmid (SELZER *et al.* 1978, 1981). By sequence comparison we know that P1 is the same promoter called P 1,4–3,9 by LIEBIG and RÜGER (1989). P 1,4–3,9 was cloned using a technique which allows very strong T4 promoters, which might otherwise be lethal for *E. coli*, to give viable transformants in this organism. It is, both theoretically and experimentally, a strong promoter in *E. coli*. It is one of a class of such promoters (group I) cloned by LIEBIG and RÜGER (1989). Two features of T4 promoters described by these authors are pertinent here. First, the information content of T4 early promoters is higher than the information content of *E. coli* promoters (SCHNEIDER *et al.* 1986). A reasonable explanation for this high information content is that these sequences supply sites for binding proteins in addition to RNA polymerase. A second intriguing property that P1 shares with a large number of other early T4 promoters (LIEBIG and RÜGER 1989) is its 6-bp inverted repeat between the −10 and −35 sequences.

Why, then, is *rIIA* not an immediate-early gene? First of all, the situation in *rIIA* is not unique. T4 promoters that are strong when cloned into *E. coli* but are used poorly, if at all, during T4 infection have been found upstream of gene *32* (BELIN *et al.* 1987; CARPOUSIS, MUDD and KRISCH 1989) and upstream of the gene *uvsX* (HINTON 1989). As has previously been discussed, this may be due to the fact that these promoters are in a supercoiled plasmid when tested in *E. coli* but in relaxed DNA when injected as part of the T4 genome (SINDEN and PETTIJOHN 1982; ALBRIGHT and GEIDUSCHEK 1986). However, it is equally possible, as discussed above, that certain T4 early promoters are in fact repressed by the action of other proteins. The P1 promoter of *rIIA* is used even when infection is carried out in the presence of chloramphenicol. Yet, little RNA is generated in such infections (DAEGELEN and BRODY 1976; YOUNG and CRONE MENARD 1980; DAEGELEN, D'AUBENTON-CARAFA and BRODY 1982; THERMES and BRODY 1984). Either its *in vivo* activity is weak compared to other promoters farther upstream, or the P1-initiated RNA is terminated before it enters the *rIIA* gene, or P1-initiated RNA is highly unstable. The DNA sequence between P1 and the *rIIA* gene does not suggest that ρ-inde-

pendent terminators exist in this region. We have probed the entire length of the *rIIA* gene by reverse transcriptase and find no evidence for differential stability of various *rIIA* transcripts. For one reason or another, P1 seems to be weak in T4 infection in comparison to upstream promoters. Is this weakness due to active repression? Such an explanation would be consistent with our data, but we have no proof that such repression exists.

This problem is related to another. Our analyses measure *amounts* of RNA in infected cells. PULITZER, COLOMBO and CIARAMELLA 1985; J. F. PULITZER, personal communication) have measured *rates* of *rIIA* RNA synthesis after infection with various mutant phages. They found that *motA*$^-$ infections did not change synthesis rates, nor did *motB*$^-$ or *motB*$^-$ *motC*$^-$ infections, but that *motA*$^-$ *motB*$^-$ double-mutant (or *motA*$^-$ *motB*$^-$ *motC*$^-$ triple-mutant) infections did diminish synthesis rates of *rIIA* RNA. This effect was about two- to threefold at 5 min after infection at 30°. We cannot correlate our results of P1, P2 and PM promoter utilization in any simple way with their measures of rates of synthesis. We do think, however, that the comparison of the two sets of results for *rIIB* RNA allows an explanation of the differences in the *rIIA* data. PULITZER, COLOMBO and CIARAMELLA (1985) found that *motA*$^-$ infections did not affect the rate of RNA synthesis for the *rIIB* gene, wheras *motA*$^-$ *motB*$^-$ or *motA*$_J$ *motB*$^-$ *motC*$^-$ infections led to a decrease in *rIIB* RNA synthesis (just as they found in their analysis of *rIIA* RNA synthesis). The *rIIB* synthesis in *motA*$^-$ infections is thought to come from upstream early promoters which synthesize *rIIB* RNA as the distal end of a polycistronic transcript (DAEGELEN and BRODY 1982; PULITZER, COLOMBO and CIARAMELLA 1985). The synthesis of rIIB protein is thought to be inefficient from this long RNA, which would explain the *motA*$^-$ sensitivity of rIIB protein synthesis (MATTSON, VAN HOUWE and EPSTEIN 1978). When we analyze *rIIB* RNA by reverse transcriptase, we find a complete *motA*$^-$ sensitivity of the two 5′ ends we detect; there is no other *motA*$^-$-insensitive 5′ end. This had previously been reported by GUILD *et al.* (1988). We conclude that the early mode *rIIB* RNA is simply not seen in our analyses because the promoters are too far upstream to be visible. If we apply analogous reasoning to our *rIIA* primer-extension data and the *rIIA* data obtained by hybridization of pulse-labeled RNA, we are led to the following model. The three promoters we have described just upstream of the *rIIA* gene are all relatively weak after infection compared to early promoters further upstream. Pulse-labeled RNA is a measure primarily of the amount of RNA transcribing into the *rIIA* gene from these upstream promoters. The *motB*, *motC* and *comC-α* effects seen on *rIIA* RNA synthesis reflect the dependencies of the utilization of these promoters and/or the ability of RNA polymerase to elongate this RNA into the *rIIA* gene.

In conclusion, *rIIA* RNA can be synthesized from three different promoters just upstream of this gene. Two are early promoters and one is a middle promoter. Nonetheless, all three of these promoters seem to yield relatively little *rIIA* RNA (at least early in infection) compared to the amount of *rIIA* RNA synthesized from promoters further upstream of the *rIIA* gene.

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