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Sizes of Bacteriophage T4 Early mRNA's Separated by Preparative Polyacrylamide Gel Electrophoresis and Identified by In Vitro Translation and by Hybridization to Recombinant T4 Plasmids

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We determined the sizes of specific T4 prereplicative mRNA's by preparative polyacrylamide gel electrophoresis, and we used the following two techniques to identify specific gene transcripts: cell-free protein synthesis accompanied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to distinguish T4 polypeptides and hybridization to recombinant plasmids containing T4 DNA of known genetic composition. In our first analysis, the use of nonsense and in-phase deletion mutants allowed unambiguous identifications of the functional transcripts that encoded genes 32, rIIB, and rIIA. In addition, we identified the functional transcripts that encoded genes 43, 45, 30, 39, and 52, the β-glucosyl transferase gene, and the deletion 293 region. A single peak of mRNA activity that coded for gp43, gp39, gprIIA, β-glucosyl transferase, and the polypeptide encoded in the deletion 293 region was present; the other polypeptides were encoded in multiple mRNA species. gp46 and gp32 were encoded by two mRNA's, and gp52 and gprIIB were encoded by three mRNA's. By hybridizing fractionated, pulse-labeled early RNA to cloned restriction fragments of T4 DNA, we identified the same specific transcripts for genes 43, 52, and rIIB. In addition, a lower-molecular-weight RNA (presumably degraded mRNA) was present even in pulse-labeled RNA preparations. The distribution of pulse-labeled RNAs that hybridized to gene 39, gene 30, gene rIIA, gene 40 plus gene 41, and gene 42 plus the β-glucosyl transferase gene indicated extensive degradation. We detected cotranscription of genes rIIA and rIIB by rehybridization of RNA first annealed to an rIIB plasmid and then eluted and annealed to an rIIA plasmid. The size distributions of normal and chloramphenicol-treated RNAs that hybridized to plasmids containing T4 immediate early gene 30, gene 39, gene 40 plus gene 41, and gene 42 plus the β-glucosyl transferase gene were not significantly different.

An understanding of the arrangement of T4 prereplicative transcription units would increase our knowledge of the regulation of T4 early genes significantly. The current model of this arrangement (28) proposes that chloramphenicol (CAM)-resistant immediate early genes (transcripts are synthesized after infection by T4 in the presence of CAM) are promoter proximal to CAM-sensitive delayed early genes (transcripts are not synthesized after infection in the presence of CAM) and that quasi-late or middle-mode genes are regulated differently than immediate early and delayed early genes. The factors that influence the transition from immediate early transcription to delayed early transcription and subsequently to middle-mode transcription are poorly understood. The *Escherichia coli* termination factor rho is thought to be involved in the shift from immediate early transcription to delayed early transcription (3, 5), and the T4 *mot* gene product is involved in the expression of at least some middle-mode genes (18, 20, 27).

Various aspects of the model summarized above have been tested. The most direct predictions are that the sizes of some immediate early transcripts would increase if delayed early transcription were allowed and that delayed early transcripts would be the distal portions of polycistronic transcripts whose promoter-proximal portions would be immediate early transcripts. This proposition was tested for the D2 region (33) and for the internal proteins IPII and IPIII, but the results were equivocal; the D2 RNA size was invariant, whereas the IP RNA seemed to be somewhat larger in the absence of CAM than the presence of CAM.

In general, the physical sizes of T4 transcripts

have been difficult to study because of the lability of the RNAs. The T4 tRNA's are an exception to this. The presumed primary transcript synthesized in vitro has been identified, and the processing of this transcript to mature tRNAs' has been studied (10). A similar study of T4 mRNA's that code for early proteins has not been performed, although such a study would clearly be relevant to any model of regulation of prereplicative transcription. In a few instances the size of the functional transcript that codes for an early protein has been identified by its ability to code for an enzymatic activity or an immunologically active polypeptide (1, 29). For the nonessential rII genes and the adjacent D region, the RNA has been identified by deletion hybridization analyses (31, 32, 39). In this case there is a discrepancy concerning the physical sizes of the rIIA and B transcripts (33, 39).

We determined the sizes of specific T4 early transcripts by polyacrylamide gel electrophoresis. The RNAs were identified after electrophoresis by the following two techniques: in vitro translation and separation of the labeled polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and hybridization of radioactive RNA to cloned restriction fragments of T4 DNA. Analyses of functional early mRNA's revealed that a number of transcripts occurred as multiple species, which indicated that there were multiple promoters or termination sites or that post-transcriptional processing occurred. Some of these same multiple transcripts could be identified by hybridization, but the radioactive RNA species corresponding to many genes were present as heterogeneous arrays of small fragments, which represented degraded fragments of the active mRNA's.

MATERIALS AND METHODS

Biochemicals and media. Most reagents were purchased from standard sources. [^{35}S]methionine was prepared as described by Crawford and Gesteland (6). Mouse RNA was a gift from Ursula Storb, and ^{3}H-labeled RNA from the midi-variant of bacteriophage Qβ (MDV RNA; 22) was a gift from Frank Ryan. The acrylamide used for gel electrophoresis was passed through a membrane filter (Millipore Corp.) before use. M9S and M9S.1 media have been described previously by Bolle et al. (2).

Bacteria, phage, and plasmids. *E. coli* B^{E}, a nonpermissive host for amber mutants, was used as a host for all infections. Plasmids were maintained in *E. coli* strain 802 (suII^{+}r$_k^{-}$m$_k^{+}$).

The bacteriophage strains with multiple mutations were constructed by standard phage crosses (Table 1). The rIIA deletion mutation EM66 and the rIIB deletion mutation 196 have been characterized by A. Bolle (personal communication). These mutations contain wholly internal in-phase deletions which correspond to losses of approximately 300 and 100 amino acids from rIIA and rIIB, respectively.

The plasmids containing T4 restriction fragments were from the collections of Mattson et al. (19) and Selzer et al. (34). The properties of these plasmids are shown in Table 2. The T4 restriction fragments in the original collection of Mattson et al. were cloned into pCR1 and were designated by the prefix pVH. Mattson and Van Houwe transferred these restriction fragments to pBR322 and gave them new designations (the 600 series), which we used. The designations of the T4 recombinant plasmids that were derived from the 600 series by cleavage with different restriction enzymes than the enzymes used in the original cloning procedure contain a suffix letter (A, B, etc). Restriction maps of the rII regions showing the origins of the DNA fragments used in this work are shown in Fig. 1 (26a, 34).

TABLE 1. *Bacteriophage T4 mutants*

Mutant	Description
32amA453 44amN82	Amino-terminal amber mutation in gene 32 (no identifiable polypeptide); no gene 44 polypeptide detected
32amA453 44amN82 rIIdel1241	rIIdel1241 covers all known point mutations in rIIA and rIIB and deletes part of the D region; no polypeptides from genes 32, 44, rIIA, and rIIB
32amH18 44amN82	18,000-dalton polypeptide from gene 32; no gene 44 polypeptide
rIIBdel196	Internal, in-phase deletion in gene rIIB making a 22,000-dalton rIIB polypeptide
rIIAdelEM66	Internal, in-phase deletion in gene rIIA making a 55,000-dalton polypeptide
32amH18 44amN82 rIIBdel196	18,000-dalton gp32; 22,000-dalton gprIIB; no gp44 identified
32amH18 44amN82 rIIBdel196 rIIAdelEM66	18,000-dalton gp32; 22,000-dalton gprIIB; 55,000-dalton gprIIA; no gp44

TABLE 2. *T4 recombinant plasmids*

Plasmid	T4 gene(s)[a]	Restriction site(s) used for cloning	Reference(s)
621	52	RI	19
622	43	RI	19
624	42, βgt	RI	19
625	30	RI	19
626A	39	*Hin*dIII	19; Young, unpublished data
627	40, 41	RI	19
pTB35	rIIA	*Hin*dIII	34
pABI	rIIA	*Hin*dIII, RI*	34; Selzer, unpublished data
pTB10	rIIAB	*Hin*dIII	34
pABIV	rIIB	RI*, *Hin*dIII	34; Selzer, unpublished data

[a] No other genes have been identified by marker rescue tests. Plasmid 621 (gene 52) contains additional DNA which has not been identified. The plasmids containing genes 43, 30, 39, rIIA (pTB35 and pABI), and rIIB (pABIV) have been shown by marker rescue tests to be wholly internal to the gene indicated. The T4 rIIA and rIIB restriction enzyme fragments used in this work are shown in the maps in Fig. 1. The length of the T4 fragment in plasmid pTB10 is 873 base pairs (26a).

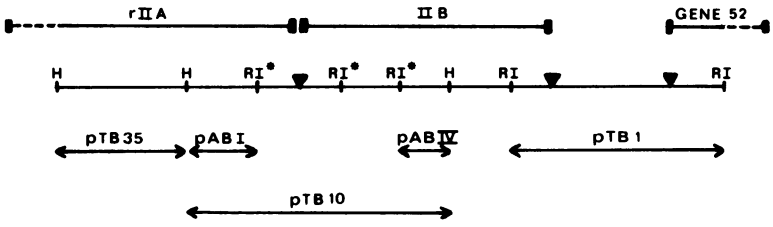

FIG. 1. *Maps of T4 rIIA and rIIB restriction enzyme fragments. The maps are drawn approximately to scale. H, HindIII.*

Nucleic acid preparation. T4 DNA was prepared from purified T4 phage, and plasmid DNA was prepared by minor modifications of the cleared lysate procedure of Katz et al. (16), as described elsewhere (42). The ethidium bromide and residual protein that remained after centrifugation in CsCl were removed by two phenol extractions.

Both radioactive RNA and nonradioactive RNA were isolated from T4-infected *E. coli* B^E cells, as described elsewhere (42).

Preparative electrophoresis of RNA on polyacrylamide gels. We used the preparative electrophoresis apparatus of Hagen and Young (14), with modifications (13, 26). Denaturation of RNA samples with dimethyl sulfoxide before electrophoresis has been described previously (13, 26). Briefly, each RNA sample dissolved in Tris-hydrochloride (pH 7.5)-EDTA was mixed with spectroscopy grade dimethyl sulfoxide so that the final concentrations in the sample were 90% dimethyl sulfoxide, 10 mM Tris-hydrochloride, and 3 mM EDTA. Then the sample was incubated for 5 min at 37°C, cooled to room temperature, and loaded onto a gel. The percent recovery of RNA activity from the gels was usually 30 to 100%.

Cell-free protein synthesis, electrophoresis of polypeptides, and densitometric analysis of autoradiograms. An S30 extract was prepared from *E. coli* MRE600 and used as described previously (26). Radioactive polypeptides were analyzed as described by Pachl and Young (26).

Hybridization to filter-bound DNA. Filters containing immobilized T4 phage or plasmid DNA were prepared and used as described elsewhere (42). If the annealed RNA was to be eluted for use in a second experiment, the first annealing was performed in 0.03 M sodium citrate-0.3 M sodium chloride (pH 7.0)-50% formamide. The RNA was eluted by heating the washed filter in a solution containing 10 mM Tris (pH 8.8) and 0.2 mM EDTA for 1 min at 100°C and then rapidly cooling the solution. The filter was removed, 50 μg of tRNA was added, the solution was incubated with 5 μg of RNase-free DNase for 15 min at 30°C and phenol extracted, and the RNA was recovered by ethanol precipitation (42). Elution of the RNA after the filters were treated with RNase was performed as described elsewhere (42).

RESULTS

Sizes of functional early transcripts. RNA was extracted 12 min after infection of *E. coli* $B^E(su^-)$ with T4 32amA453 44amN82, as described above. (Amber mutants were designated by gene number, followed by allele designation; for example, the amber mutation A453 is in gene 32, and the mutant containing this mutation was designated 32amA453.) About 400 μg of purified RNA was denatured by treatment with 90% dimethyl sulfoxide before continuous-elution preparative electrophoresis on a polyacrylamide gel (13) (Fig. 2). The radioactive polypeptides synthesized in vitro by the fractionated RNA were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The identification of rIIB mRNA activity was facilitated by using phage containing nonsense mutations in genes 32 and 44 and infecting an su^- host. This allowed gprIIB to be identified and quantitated in the absence of comigrating gp32 and gp44 (23). (gprIIB, gp32, and gp44 are the polypeptide products of genes rIIB, 32, and 44, respectively.) Using the double amber mutant had an additional advantage. Because both gp32 and gp44 were required for DNA synthesis and hence late T4 RNA synthesis, this mutant did not make any late RNA which could interfere with the identification of early mRNA species. Figure 2 shows an autoradiogram of the dried gel.

Without considering the identity of the individual proteins synthesized by the fractionated RNA, we observed two different types of behavior for the mRNA species identified by in vitro translation. Some mRNA's migrated at one or more unique rates, as shown by the presence of mRNA activities for their cognate proteins in one or more unique sets of adjacent fractions collected from the gel. Other mRNA's appeared to be polydisperse; their activities were spread rather continuously across the gel. For some RNA species this "tailing" appeared to result from a major peak whose mRNA activity trailed throughout the remaining fractions. However, in other cases the mRNA activity was distributed fairly uniformly over a broad range of molecular weights. The polydispersity could have arisen for one or more reasons, including incomplete washing from the elution chamber, aggregation of the RNA, physical size heterogeneity of the mRNA, and comigration of different polypep-

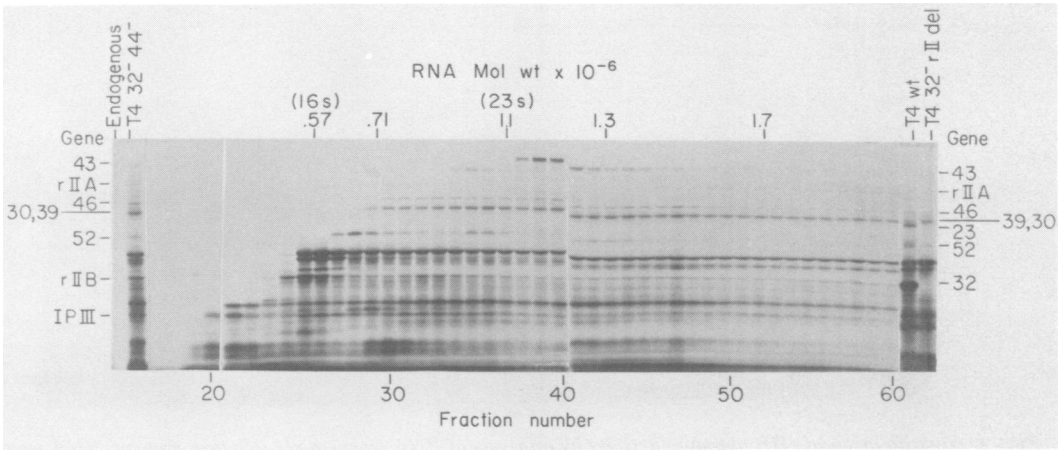

FIG. 2. *Autoradiogram of T4 early polypeptides synthesized by fractionated 32⁻ 44⁻ RNA. A 400-μg sample of RNA isolated 12 min after infection of E. coli B^E with T4 32amA453 44amN82 was denatured with 90% dimethyl sulfoxide for 3 min at 37°C and then fractionated on a preparative gel (diameter, 5 cm) containing 2.25% acrylamide. The RNA was electrophoresed at 15 mA for 11 h, and the eluted RNA was concentrated by ethanol precipitation and then translated in a cell-free protein-synthesizing system containing [35S]methionine. The radioactive polypeptides were separated by electrophoresis on a discontinuous sodium dodecyl sulfate gel system containing equal volumes of 12.5, 10, and 7.5% acrylamide layered sequentially over one another. The stacking gel contained 3% acrylamide. After electrophoresis for 3 h at 25 mA, the gel was treated with dimethyl sulfoxide and subjected to fluorography as described in the text. The polypeptides synthesized in vitro from unfractionated RNAs derived from cells infected with phages of the genotypes indicated above the slots are shown at the sides to allow identification of specific T4 polypeptides. The molecular weight scale at the top was derived from the 16S and 23S rRNA's present on this gel and from these and additional molecular weight standards separated on an identical gel (see Fig. 8). The two gels used in this analysis did not separate the T4 polypeptides identically. gp43 was the upper band in both gels. wt, Wild type.*

tides encoded by mRNA's of different but overlapping sizes. For those mRNA's which had an intense peak of activity followed by trailing, we assumed that the apparent heterogeneity was primarily an artifact caused by incomplete elution from the elution chamber and efficient translation of the late-eluting fractions in the cell-free system, as has been observed with T7 mRNA (25, 26).

Several proteins could be identified readily after translation of the fractionated RNA, based on their relative electrophoretic mobilities, their known molecular weights, and analyses of amber mutants (24, 40). The autoradiogram in Fig. 2 shows the genes that coded for these proteins; they included gene 43, gene 46, gene 30 plus gene 39, gene 52, gene 32, gene rIIB, and gene IPIII. gp30 and gp39 comigrated on the gel shown in Fig. 2; in other analyses (see Fig. 6) these gene products were separated from each other, and their individual mRNA activities could be identified. The RNA for rIIA could not be identified unambiguously. There were two reasons for this. gprIIA was synthesized poorly in vitro, and two different mRNA fractions coded for a protein that was approximately the same size as gprIIA. These two mRNA species were identified in fractions 34 to 36 and 39 to 60 of the autoradiogram shown in Fig. 2. The protein encoded by the mRNA that eluted in fractions 34 to 36 had the same mobility as the polypeptide that was encoded by the deletion 293 (del 293) region (24). gprIIB appeared to be identified unambiguously on the gel since no gp32 was made when unfractionated RNA was used (Fig. 2). However, even partial suppression of the amino-terminal gene 32 amber mutation (A453) could cause synthesis of enough gp32 to allow it to be confused with gprIIB. To overcome this problem and to identify the mRNA for rIIA, we performed two experiments. In the first experiment RNA from the triple mutant T4 32amA453 44amN82 rIIdel1241 was analyzed after fractionation on a preparative polyacrylamide gel. In this mutant both rIIA and rIIB were deleted completely. The translation products were analyzed as described above (Fig. 3). No rIIB polypeptide was found on the gel, whereas most of the other polypeptides with molecular weights less than 60,000 which were present on the Fig. 2 gel were identified, and their mRNA's migrated at approximately the same rates as on the gel shown in Fig. 2. An exception to this was the gene 52 message activity (see below). This

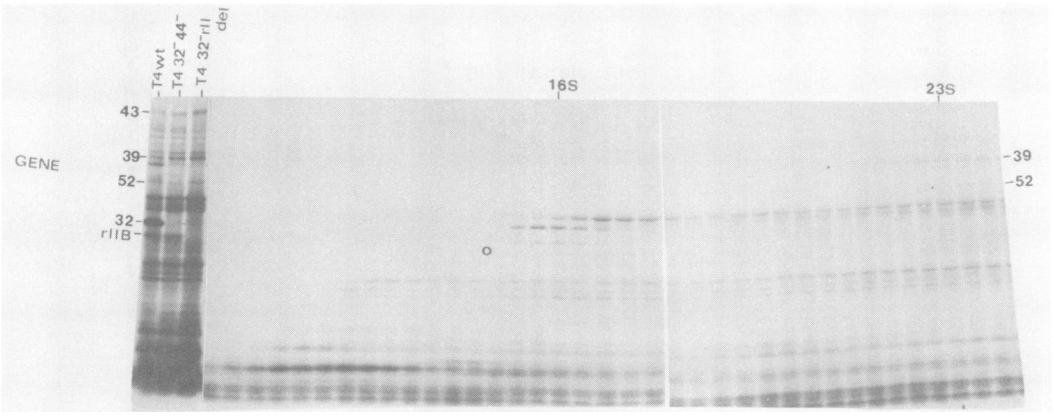

FIG. 3. *Identification of rIIB message activity by analysis of RNA from an rII deletion mutant. RNA was extracted 12 min after infection of E. coli* B^E *by T4 32amA453 44amN82 rIIdel1241 and analyzed as described in the legend to Fig. 2 and in the text. The polypeptides synthesized in vitro by unfractionated RNA isolated 12 min after infection by wild-type T4 (T4 wt) T4 32amA453 44amN82, and T4 32amA453 44amN82 rIIdel1241 are shown in the three slots on the left. The elution positions of the 16S and 23S rRNA markers are indicated on the top of the autoradiogram. The open circle on the figure indicates the expected position of the rIIB polypeptide.*

was additional evidence that the polypeptide labeled gprIIB in Fig. 2 was the product of the rIIB gene. Unfortunately, no polypeptides with molecular weights of more than 60,000 were made in the second experiment. Thus, the presumptive rIIA message in Fig. 2 could not be identified because neither of the two potential rIIA polypeptides was synthesized.

Only the middle-sized (M_r, 1.2 × 10^6) gp52 message activity was present in the RNA isolated after infection with the deletion mutant rII1241, whereas three peaks of gp52 message activity were detected when RNA from wild-type T4 was analyzed. The right endpoint of del1241 was estimated to be 0.7 kilobase from the right or 3′ end of rIIB (7, 8). Since it has been estimated that rIIB and gene 52 are separated by about 3.5 kilobases (7, 40), the right endpoint of del1241 is about 2.8 kilobases from gene 52. None of the gene 52 messages observed was large enough to start within this region and continue through gene 52.

A more positive identification of rIIB mRNA and identification of rIIA mRNA were achieved by using in-phase deletion mutants. del196 is an internal, in-phase deletion within rIIB that produces a shortened polypeptide with a molecular weight of 22,000 (A. Bolle, personal communication). delEM66 lies within rIIA, and coding also remains in phase in this case, so that an rIIA polypeptide with a molecular weight of about 55,000 is synthesized (Bolle, personal communication). These mutations were introduced into the double amber mutant 32amH18 44amN82 to produce triple and quadruple mutants with the following genotypes: T4 32amH18 44amN82 delEM66, T4 32amH18 44amN82 del196, and T4 32amH18 44amN82 del196 delEM66. The gene 32 amber allele used for these experiments (H18) had the advantage that it produced a recognizable amber fragment with a molecular weight of 18,000, so the gene 32 message could also be identified. Figure 4 shows the identification of these polypeptides on a sodium dodecyl sulfate-polyacrylamide gel. The truncated gene 32, rIIA, and rIIB polypeptides were identified easily and migrated in regions of the gel that were free of other radioactive polypeptides, as shown by the samples isolated from the wild-type and mutant-infected cells.

RNA from the triple mutant 32^- 44^- rIIB del196 was fractionated by preparative polyacrylamide gel electrophoresis essentially as described in the legend to Fig. 2. The eluted RNA was translated in a cell-free protein-synthesizing system, and the radioactive polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure 5 shows an autoradiogram of the dried gel. The rIIB del196 polypeptide and the gene 32 amber fragment H18 could be identified easily. The rIIB del196 message activity migrated more rapidly than the wild-type rIIB message activity, as expected. This was evident by the relative positions of the first rIIB message peaks and the position of the 16S rRNA (Fig. 2 and 5). The molecular weights and the differences between wild type and del196 are discussed below. gprIIA appeared to be present in fractions 68 to 74, which also contained a small peak of rIIB message activity. Gene 43 message activity migrated slightly faster than the putative polycistronic rIIA-B message. How-

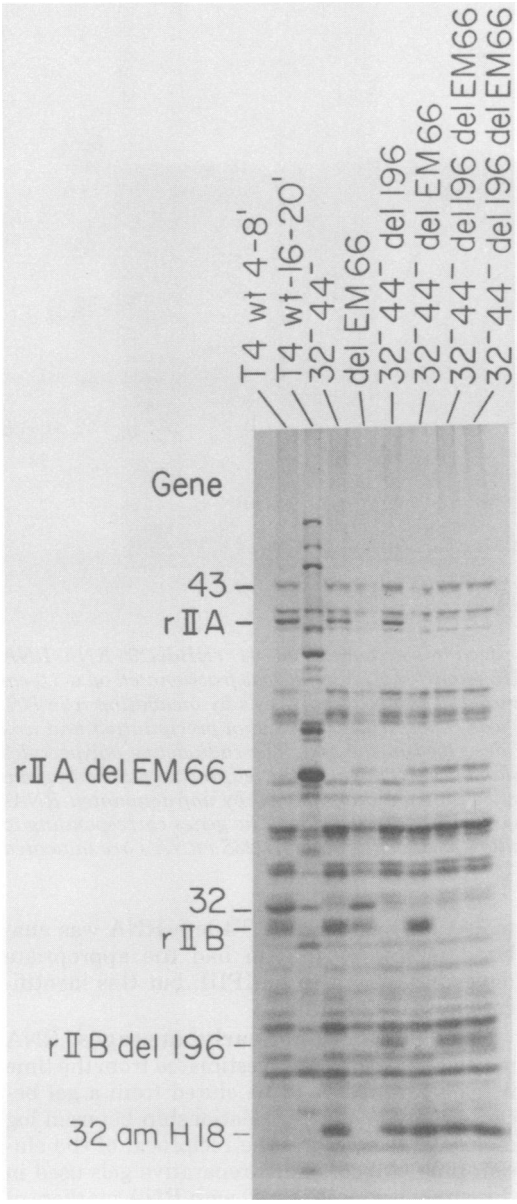

FIG. 4. *T4 polypeptides synthesized in vivo by using wild-type (wt) and mutant phage. Cultures of E. coli B^E infected with T4 phage were labeled with [^{35}S]methionine at either 8 to 12 min or 20 to 24 min (infection with wild-type phage only [slot labeled T4 wt-16-20']). The radioactive polypeptides were extracted and separated on a 10% polyacrylamide by electrophoresis for 5 h at 25 mA. The relevant genotypes are shown at the top; the genes corresponding to the marked polypeptides are shown on the left.*

ever, there was a considerable amount of background due to translation of endogenous mRNA's in the S30 extract, which interfered with the identification of minor T4 polypeptides, such as rIIA. Most of the other T4 mRNA's identified in Fig. 2 could also be identified in Fig. 5, and the migration rates of these mRNA's were approximately the same on the two gels. Some of these messages are identified by gene number (Fig. 5).

A shortened rIIA polypeptide should be synthesized more readily in the cell-free system, thereby making the rIIA message easier to identify. To do this, RNA extracted from cells infected with the quadruple mutant 32^- 44^- del196 delEM66 was fractionated, and the mRNA activity was analyzed as described above. The rIIB del196, rIIA delEM66, and 32amH18 polypeptides were identified easily. The mRNA activities for rIIA delEM66 and rIIB del196 polypeptides both comigrated at a rate that represented an RNA with a molecular weight of approximately 1.1×10^6. The rIIB del196 message activity was also present in lower-molecular-weight fractions, as observed in Fig. 2 and 5. No other message activity for rIIA delEM66 was detected. An analysis of the distribution of gene 32, rIIA, and rIIB message activities is presented below.

Quantitation of functional early messages. To compare the amounts of protein-synthesizing activity in the different mRNA's coding for the same polypeptide and to examine the apparent comigration of rIIA and rIIB message activities more carefully, autoradiograms of gels such as the one shown in Fig. 2 were scanned with a microdensitometer. The results of these measurements are shown in Fig. 6 for genes 43, del293, 52, rIIB, 46, 30, and 39 (from Fig. 2 and other data not shown) and in Fig. 7 for genes rIIBdel196 and rIIAdelEM66 (from the analysis of RNAs extracted from cells infected with the mutant 32^- 44^- rIIBdel196 rIIAdelEM66). Of these nine genes, only genes 43, del293, 39, and rIIA were represented by single messages with unique sizes. There were two peaks of message activity for genes 32 and 46 and three peaks of message activity for genes rIIBdel196 and 52. The largest RNA from the rIIB region was not detected in the RNA isolated from $rIIB^+$-infected cells (Fig. 6). Gene 30 message activity migrated heterogeneously, with no apparent well-defined peak of activity. gp30 and gp39 were not separated on the gel shown in Fig. 2; thus, it appeared that the gene 39 message activity was also heterogeneous. Numerous other message activities having one of these types of behavior were observed (Fig. 2, 3, and 5). The genetic origins of most of these were not identified. A conspicuous protein having a molecular weight of 20,000 to 25,000 was translated from four different messengers having molecular weights of 0.25×10^6, 0.50×10^6, 0.8×10^6, and 1.0×10^6. We found a very similar distribution of messenger activity for a protein that had an identical

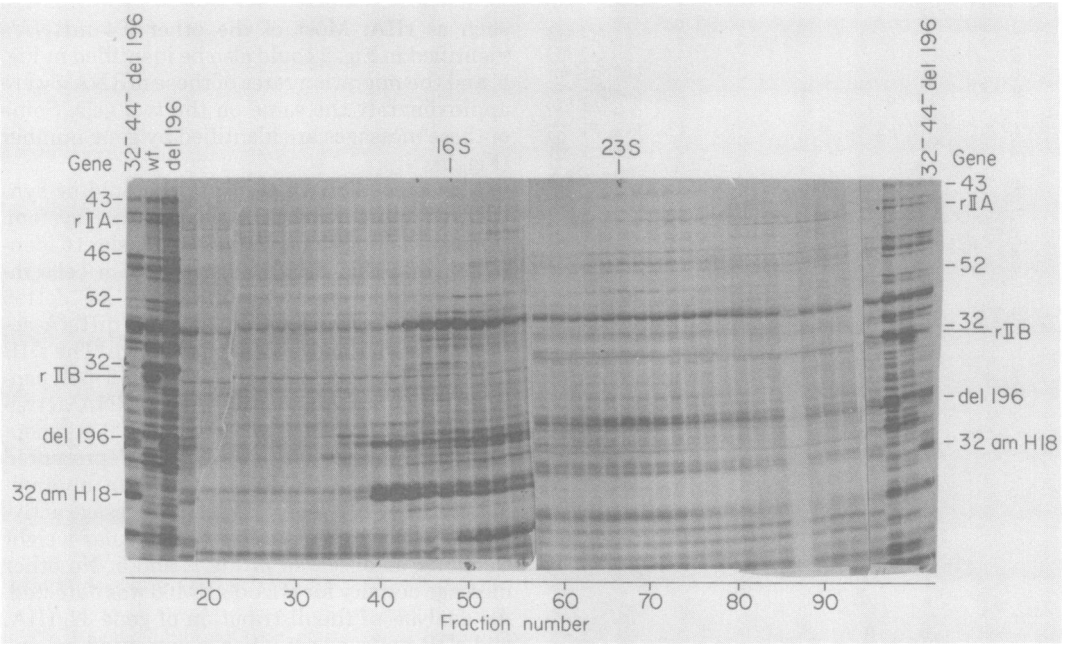

FIG. 5. *Autoradiogram of T4 early polypeptides synthesized by fractionated 32⁻ 44⁻ rIIBdel196 RNA. RNA isolated 12 min after infection of E. coli B^E by T4 32amH18 44amN82 rIIBdel196 was fractionated on a 5.0-cm 2.0% acrylamide–0.5% agarose gel. The RNA was denatured before electrophoresis by incubating it in 80% dimethyl sulfoxide for 10 min at 37°C. The eluted RNA was concentrated by ethanol precipitation and was translated in a cell-free protein-synthesizing system as described in the text. The radioactive polypeptides were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel. The gel was dried, and an autoradiogram was made. The slots at the sides contained the radioactive proteins programmed by unfractionated RNAs isolated after infection of E. coli B^E by the phage strains shown above the slots. The genes corresponding to the marked polypeptides are shown on the sides. The elution positions of 16S and 23S rRNA's are indicated at the top. wt, Wild type.*

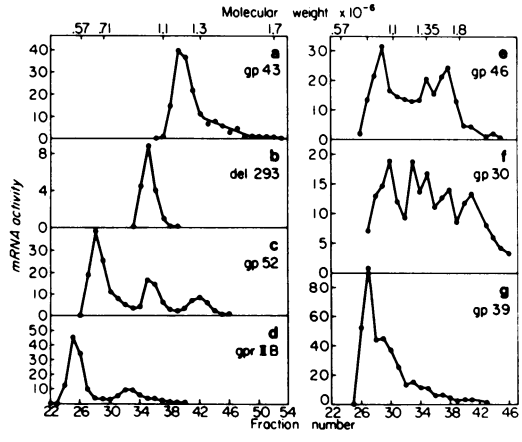

FIG. 6. *Densitometric analyses of T4 mRNA activities. Autoradiograms such as those shown in Fig. 2, 3, and 5 were scanned with a Joyce-Loebl double-beam recording microdensitometer equipped with a model JL-20013 integrator. The exposures chosen for analysis provided a linear response between film darkening as measured on the recorder and radioactivity in the sample. The gene products analyzed are indicated on the figure.*

molecular weight when T4 late RNA was analyzed (42). This protein had the appropriate molecular weight to be IPIII, but this identification was not verified.

Sizes of functional early messages. RNA molecular weight can be estimated from the time it takes an mRNA to be eluted from a gel because there is a linear relationship between log molecular weight and the reciprocal of the elution time (13, 26). The preparative gels used in this study were calibrated with RNA markers of known molecular weights, ranging from a tRNA with a molecular weight of 2.5×10^4 to 28S rRNA from mouse liver cells (molecular weight 1.7×10^6). These markers defined a curve from which the molecular weights of RNAs of unknown size could be determined. Figure 8 shows a curve for marker RNAs fractionated on a preparative gel containing 2.25% polyacrylamide. Except for tRNA, all of the markers lay on a straight line. Most of the RNAs identified migrated in the linear region. The molecular weights of the T4 mRNA's identified above are shown in Table 3, together with the molecular weights of the corresponding polypeptides. Fig-

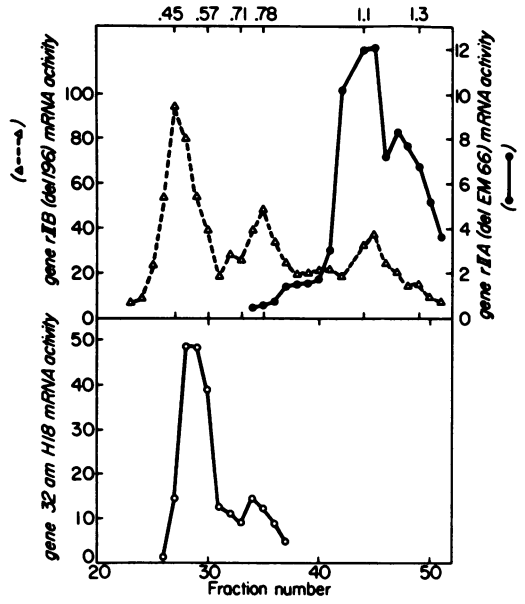

FIG. 7. *Densitometric analyses of rII mRNA activities. RNA isolated after infection of E. coli B^E by T4 32amH18 44amN82 rIIBdel196 rIIAdelEM66 was separated on a 2.25% polyacrylamide gel. The RNA was not denatured with dimethyl sulfoxide. After electrophoresis and elution, the RNA was analyzed as described in the legend to Fig. 2 and in the text. The radioactive polypeptides were separated on a 10% polyacrylamide gel, from which an autoradiogram was made. The rIIBdel196, rIIAdelEM66, and 32amH18 polypeptides were identified by comparison with the corresponding polypeptides synthesized by unfractionated RNA, which were present on the same gel. Densitometric analyses of these three polypeptides were performed as described in the legend to Fig. 6. The RNA molecular weight scale at the top was derived from an identical gel on which a mixture of yeast and E. coli RNAs was fractionated.*

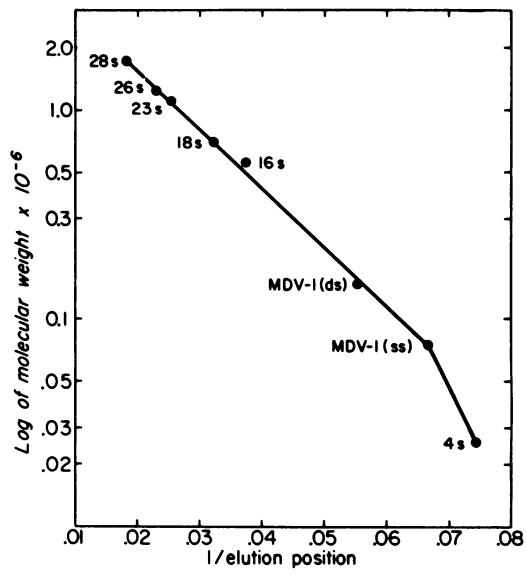

FIG. 8. *Plot of logarithm of molecular weight versus the reciprocals of the elution positions of the RNA standards. A mixture containing double-stranded ^{3}H-labeled Qβ MDV-1 RNA and nonradioactive mouse, yeast, and E. coli RNAs was denatured with dimethyl sulfoxide and separated by electrophoresis at 15 mA on a 2.25% polyacrylamide gel. The elution positions of the radioactive RNA species were determined by counting a portion of each sample in a liquid scintillation counter. The elution positions of the nonradioactive rRNA's were determined with a chart recorder monitoring the elution buffer. Molecular weights of 1.7×10^6, 1.3×10^6, 1.07×10^6, 0.7×10^6, 0.56×10^6, 0.148×10^6, and 0.074×10^6 were assumed for mouse 28S, yeast 26S, E. coli 23S, mouse 18S, E. coli 16S (17), Qβ MDV-1 double-stranded (ds) and Qβ MDV-1 single-stranded (ss) RNAs (22), respectively. The double-stranded Qβ MDV-1 RNA, which was present in small quantities, presumably represented nondenatured molecules.*

ures 2, 3, and 5 contain molecular weight scales, from which the molecular weights of other RNA species could be determined. Table 3 also shows the molecular weight of the mRNA that coded for T4 β-glucosyl transferase (designated mβgt); this information was obtained by measuring β-glucosyl transferase enzyme activity after RNA fractionated by preparative gel electrophoresis was translated (data not shown). This RNA was extracted from cells infected with a T4 mutant containing a defective α-glucosyl transferase to avoid having to distinguish between the two enzyme activities.

Table 3 shows the excess coding capacities of the genes and thus provides an indication of whether the mRNA's were monocistronic or polycistronic. The excess coding capacity of the polycistronic rIIAB transcript was not rII-coding capacity. The size of this polycistronic RNA decreased with each rII deletion introduced into the phage, and the excess coding capacity remained approximately constant, indicating a roughly quantitative agreement between the size of the rII deletion and the decrease in size of the polycistronic RNA.

Sizes of pulse-labeled early transcripts. The isolation and characterization of cloned fragments of T4 DNA (19, 34, 37, 38) provided a convenient source of specific hybridization probes for T4 messages (42). By hybridizing pulse-labeled, fractionated RNA to filters containing early genes, the sizes of both the functional and the nonfunctional transcripts could be measured. By annealing specific gene fragments with pulse-labeled RNA rather than with continuously labeled or unlabeled RNA, we hoped to detect full-length transcripts.

Radioactive T4 early RNA was prepared as

TABLE 3. *RNA and polypeptide molecular weights of T4 mRNA activities*

Gene	mRNA designation	M_r of RNA ($\times 10^6$)	M_r of polypeptide[a]	Excess coding capacity of mRNA (no. of amino acid residues)
43	m43	1.2	112,000	200
del293	mdel293	1.0	103,000	70
46	m46a	0.95	71,000	300
	m46b	1.5	71,000	850
39	m39	0.82	64,000	240
52	m52a	0.65	51,000	200
	m52b	1.0	51,000	550
	m52c	1.3	51,000	850
32	m32a	0.52	36,000	200
	m32b	0.75	36,000	430
βgt	mβgt	0.67	46,000	250
rII transcripts				
rIIAB polycistronic[b] $rIIA^+$ $rIIB^+$		1.5	86,000 + 33,000	420
$rIIA^+$ rIIBdel196		1.3	86,000 + 22,000[c]	320
rIIAdelEM66 rIIBdel196		1.1	55,000 + 22,000[c]	400
$rIIB_a^+$		0.55	33,000	250
$rIIB_b^+$		0.88	33,000	580
$rIIBdel196_a$		0.45	22,000	250
$rIIBdel196_b$		0.78	22,000	580

[a] See references 24 and 39.

[b] The excess coding capacity is non-(rIIA + rIIB).

[c] Bolle, personal communication. The M_r of gprIIA was smaller than the value of 95,000 reported by O'Farrell et al., (24), but it agreed with the data reported here.

described elsewhere (42) and was fractionated by electrophoresis on continuous-elution preparative polyacrylamide gels as described above. The fractionated RNA was hybridized to nitrocellulose filters containing immobilized plasmid DNA. Each filter contained DNA from a plasmid containing a single T4 restriction fragment of known genetic origin. In some cases, restriction fragments wholly internal to a gene were used so that the transcript could be associated unambiguously with one gene. In other cases, the restriction fragment used as a hybridization probe contained parts of two adjacent genes. In these cases, the transcripts could come from one gene or from both genes.

Figure 9 shows the results obtained by performing hybridizations to plasmids containing gene 43, gene 52, gene rIIB, gene rIIA, gene 39, gene 40 plus gene 41, gene 42 plus the β-glucosyltransferase gene (designated βgt), and gene 30. Significant fractions of the RNase-resistant radioactivity representing the RNAs from gene 43, gene 52, gene 42 plus gene βgt, and gene rIIB (Fig. 8a through c and g) had the same molecular weights as the functional mRNA's described above. For example, there was a peak of RNA activity that hybridized to an internal fragment of gene 43 which had a molecular weight of 1.1×10^6. However, most of the RNA complementary to gene 43 was much smaller and migrated heterogeneously at molecular weights ranging from 0.1×10^6 to 0.8×10^6. A portion of the gene 52 message migrated in discrete peaks with molecular weights of 0.60×10^6 and 0.85×10^6. The sizes of these two species were similar to the sizes of two of the three functional gene 52 messages (Table 3). rIIB RNAs that migrated as discrete peaks with molecular weights of 0.50×10^6 and 0.80×10^6 also corresponded closely in size to the two lower-molecular-weight rIIB messages detected by in vitro translation. More than 50% of the gene 52 and rIIB messages were smaller than the discrete peaks observed, as was the case for gene 43.

A polycistronic rIIAB message was detected by in vitro translation (Fig. 6). We thought that this would be a major species in RNA that was pulse-labeled at an early time since it is believed to be the initial transcript for rIIB (31). However, no rIIB RNA that corresponded in molecular weight to the rIIA-B polycistronic RNA detected by in vitro translation was resolved (Fig. 9c). Hybridization to a plasmid containing an internal *Hin*dIII restriction fragment or rIIA also failed to detect a large rIIA transcript (Fig. 9d). In fact, we detected very little RNA complementary to rIIA, probably because there was less rIIA RNA than RNAs for the other early genes studied (41). RNAs complementary to gene 39, gene 40 plus gene 41, gene 42 plus gene

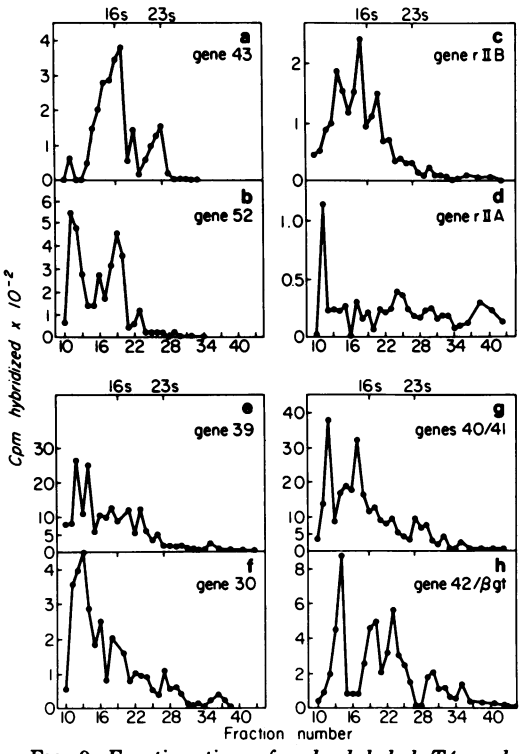

FIG. 9. *Fractionation of pulse-labeled T4 early RNA and detection of specific T4 RNAs by hybridization to recombinant plasmids. RNA was labeled with [^{3}H]uracil between 3 and 6 min after infection of E. coli B^E by wild-type T4 as described in the text. The labeling was terminated at 6 min by adding cold uracil and KCN, and the cells were rapidly disrupted by hot sodium dodecyl sulfate lysis (14). About 50 μg of RNA containing 5 × 10^6 cpm was subjected to electrophoresis on a 2.25% polyacrylamide gel as described in the legend to Fig. 2 and in the text. The RNA was not treated with dimethyl sulfoxide. Fractions (1 ml; 20 min of elution time at a flow rate of 0.05 ml/min) were collected. A small portion of each sample was precipitated with trichloroacetic acid to determine the distribution of total radioactive RNA, and 0.50-ml portions of fractions 10 through 45 (representing RNA species in the molecular weight range from 0.025 × 10^6 to 2 × 10^6) were incubated for 18 h at 67°C in siliconized scintillation vials containing plasmid DNA immobilized on nitrocellulose filters (see text). After 18 h, the scintillation vials were chilled rapidly, and the filters were removed, washed several times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), treated with RNase, washed several more times with 2× SSC, dried, and counted with a liquid scintillation counter. The background radioactivity that hybridized to a control filter which was present in each vial and contained only pBR322 DNA was subtracted. This value ranged from 99 cpm in fraction 10 (the peak of total radioactivity eluted from the gel) to 30 cpm in fractions containing high-molecular-weight RNA (molecular weight, >10^6). The T4 recombinant plasmids used in the hybridizations and the T4 genes which they contained were as follows: p622 containing gene 43 (a); p621 containing gene 52 (b); containing pABIV containing gene rIIB (c); pABI containing gene rIIA (d); p626A containing gene 39 (e); p627 containing genes 40 and 41 (f); p624 containing genes 42 and βgt (g); p625 containing gene 30 (h).*

βgt, and 30 migrated heterogeneously (Fig. 9e through h). The molecular weights of the RNAs that hybridized to these genes ranged from 2.5 × 10^4 to about 1.5 × 10^6. There were some high points in the distributions of specific RNAs for gene 39, gene 40 plus gene 41, gene 42 plus gene βgt, and gene 30, but most of the peaks were not reproducible when a different RNA preparation was analyzed. The peak of mRNA that was complementary to gene 42 plus gene βgt in fractions 18 through 20, which represented an RNA molecular weight of 0.6 × 10^6, was the same size as the mRNA that coded for β-glucosyl transferase in vitro (Table 3; unpublished data).

Sizes of pulse-labeled early transcripts after denaturation with glyoxal. Electrophoresis of RNAs completely denatured by glyoxal (21) provided better estimates of RNA molecular weights than analysis of native RNAs and should have diminished artifacts caused by RNA-RNA aggregation. Radioactively labeled T4 RNA was treated with glyoxal and fractionated by electrophoresis as described previously. The eluted RNA was hybridized to four different plasmids containing restriction fragments of genes rIIA, rIIB, 43, and 52. Control experiments showed that glyoxalated RNA hybridized with the same efficiency as non-glyoxalated RNA, probably because the glyoxal adducts were unstable and came off the RNA at the elevated temperature used for hybridization (Fig. 10).

Glyoxalated RNAs complementary to genes rIIA, rIIB, 43, and 52 migrated more heterogeneously than the native RNAs. However, we observed the same range of molecular weights with the denatured RNA as with the native RNA (which was denatured with dimethyl sulfoxide before electrophoresis). Gene 43 RNA migrated very heterogeneously. The distribution of RNA that hybridized to gene 43 extended to a maximum molecular weight of approximately 1.3 × 10^6: this was similar to the value of 1.1 × 10^6 obtained when native RNA was used.

Denaturation with glyoxal increased the heterogeneity of the migration rates of all four RNAs examined; rIIB RNA was the least affected. In other experiments we observed similar increased heterogeneity with other early RNAs (complementary to plasmids containing gene 30, gene 40 plus gene 41, gene 42 plus gene βgt, and gene 39). Nevertheless, the same discrete RNA

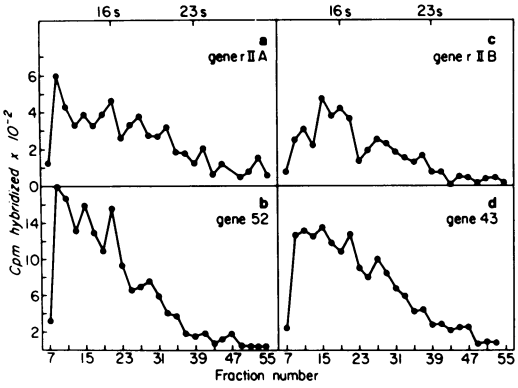

FIG. 10. *Fractionation of pulse-labeled T4 early RNA denatured by glyoxal and detection of specific T4 RNAs by hybridization to recombinant plasmids. The RNA preparation described in the legend to Fig. 9 was analyzed in an identical manner, except that the RNA was treated with 1 M glyoxal–50% dimethyl sulfoxide in 10 mM KPO_4 buffer (pH 7.0) for 60 min at 50°C before electrophoresis on a 2.0% acrylamide–0.5% agarose gel. (a) pABI (gene rIIA). (b) p621 (gene 52). (c) pABIV (gene rIIB). (d) p622 (gene 43).*

species was often discerned, suggesting that RNAs with these molecular weights existed and were not due to aggregation.

Cotranscription of rIIA and rIIB. Schmidt et al. (31) detected cotranscription of rIIA and rIIB by using deletion hybridization techniques. We detected comigration of mRNA activity for rIIA and rIIB polypeptides but could not reproducibly detect radioactive rIIA or rIIB RNA of this molecular weight. The failure to find radioactive rIIA or rIIB RNA that migrated at the expected position of a polycistronic rIIA-rIIB message could have been due to rapid breakdown of full-length transcripts. In an attempt to detect linked rIIA-rIIB transcripts, we used plasmids containing restriction fragments from the rII region (Table 2) (35, 39). Radioactive T4 early RNA was hybridized to pTB17, which contains a restriction fragment that includes the 3′ end of rIIB, the 5′ end of gene 52, and the region in between which is not deleted by saΔ9 (7). After hybridization to filters containing pTB17 DNA, the RNA was eluted and hybridized to either a pTB17 filter or a filter containing pTB35 DNA. pTB35 contained an internal rIIA restriction fragment. We could not reproducibly detect linked rIIA-B transcripts when this procedure was used, although the eluted RNA hybridized efficiently to pTB17 a second time. One possible explanation for this failure was the small amount of polycistronic rIIAB transcript compared with the smaller but more abundant rIIB transcripts (Fig. 6), which would have completed more effectively for the complementary DNA in the initial hybridization. However, when we hybridized the RNA first to pTB35 and then to pTB17, we also failed to detect linked rIIAB transcripts.

The most likely reason for the failure to detect linked rIIAB transcripts with pTB35 and pTB17 was that there were very few intact polycistronic transcripts. The T4 DNA fragments in pTB35 and pTB17 are separated in the T4 genome by about 0.9 kilobase. A cleavage of the polycistronic rIIAB RNA anywhere in this region, either in vivo, during isolation, or during the first hybridization, would have unlinked the rIIA- and rIIB-specific RNAs. If this reasoning is correct, linked rIIAB transcripts might have been detected more easily by using rIIA- and rIIB-specific DNA fragments that were more closely linked on the T4 genome. Selzer (unpublished data) obtained recombinant DNA clones from the rIIAB junction region by partial RI* digestion of a *Hin*dIII fragment spanning the rIIAB junction region. The recombinant clones obtained by this procedure included a clone which had only rIIA sequences (pABI) and another clone which had only rIIB sequences (pABIV) on the plasmid. Recently, this region was sequenced (26a), and a promoter sequence (Pribnow box) and the presumed start site of the rIIB middle-mode RNA were located in a DNA region of rIIA that lie between the two fragments cloned in pABI and pABIV. These two plasmids should hybridize only to an rIIA-specific RNA and an rIIB-specific RNA, respectively, because pABI lies to the left (5′) of the rIIB middle-mode promoter.

Cotranscription of rIIA and rIIB was detected by rehybridizing RNA first hybridized to pABI or pABIV and then subjecting the preparation to a second annealing. In the second hybridization reaction radioactive RNA that had been eluted from pABI was able to hybridize to pABIV, and RNA that had been eluted from pABIV was able to hybridize to pABI (Table 4). The control experiment with RNase-treated hybrids showed that the abilities of RNAs recovered from pABI and pABIV to hybridize to pABIV and pABI, respectively, depended on RNA sequences that were not in a DNA-RNA hybrid form during the first annealing. None of the radioactive RNA that was eluted from pABI and pABIV was able to hybridize in the second annealing to pABIV and pABI, respectively, if the first hybrids were treated with RNase, although the RNase-treated hybrids did yield RNAs that rehybridized to plasmids containing sequences identical to those used in the first annealing. An additional control showed the specificity of hybridization. The same low level of background radioactivity was observed with

TABLE 4. *Cotranscription of rIIA and rIIB*

Plasmid used in first annealing[a]	RNase treatment after first annealing[b]	Input for second annealing (cpm)	Amt (cpm) annealed in second hybridization with:				% Reannealed to filter containing:[c]	
			pABI (rIIA)	pABIV (rIIB)	p661 (gene 12)	pBR322	rIIA	rIIB
pABI(rIIA)	+	416	134	30	21	32	25	0
pABI(rIIA)	−	1,460	551	275	30	46	36	17
pABIV(rIIB)	+	290	30	110	26	24	0	27
pABIV(rIIB)	−	408	116	224	24	35	21	48

[a] The initial input radioactivity was approximately 10^6 cpm of [^{3}H]RNA labeled from 3 to 6 min after infection by wild-type T4 at 30°C, and hybridization was for 30 min at 40°C in a final volume of 150 μl containing 50% formamide, 0.03 M sodium citrate, and 0.3 M sodium chloride.

[b] Each second hybridization reaction mixture was incubated for 72 h under the conditions described in footnote *a* for the initial hybridization. The hybrids were not treated with RNase after the second reaction.

[c] A background of 30 cpm was subtracted from all raw data.

filters containing either the vector alone (pBR322) or a plasmid containing unrelated T4 gene 12.

The relative amounts of the polycistronic rIIAB transcript and the non-polycistronic rIIB transcript could be estimated from the data in Table 4. It appeared that at least 50% of the eluted rIIA transcripts extended into rIIB and that about 50% of the rIIB transcripts labeled between 3 and 6 min after infection included rIIA sequences; that is, they were transcribed from the rIIA promoter. These values were very rough estimates since the hybridization efficiency in the first annealing was unknown and in the second annealing not all of the RNA present was recovered as hybrid.

Sizes of immediate early transcripts synthesized in the presence and absence of CAM. T4 protein synthesis is required in vivo for the synthesis of all prereplicative transcripts (12, 30). The average sizes of IPII and IPIII transcripts made in the presence of CAM were less than the sizes of these transcripts made in the absence of CAM (1). This was interpreted to mean that CAM prevented these transcripts from being elongated into neighboring genes. An alternative interpretation was that CAM caused a breakdown of the RNA so that transcripts shorter than full-length transcripts were detected. The data discussed above indicated that many T4 RNAs were present primarily in transcripts shorter than full-length transcripts even in the absence of CAM.

We examined the sizes of normal transcripts and transcripts from cells (CAM RNA) CAM-treated from immediate early gene 30, gene 39, gene 52, gene 42 plus gene βgt, and gene 40 plus gene 41 by hybridizing fractionated RNAs to the appropriate plasmids. Other experiments (41) had shown that plasmids containing these genes hybridized to T4 RNA synthesized in the presence of CAM. Our results are shown in Fig. 11. The RNAs were glyoxylated before electrophoresis to prevent aggregation and to provide more reliable estimates of molecular weights. As noted above, denaturation of normal T4 RNA led to a more heterogeneous migration pattern. The molecular weight distributions of the complementary normal and CAM RNAs were distinctly different only for gene 40 plus gene 41. The molecular weight of the CAM RNA for gene 40 plus gene 41 was less than the molecular weight of the normal RNA for gene 40 plus gene 41. However, the sizes of CAM RNAs synthesized from gene 30, gene 39, and gene 42 plus gene βgt were not different than the sizes of the normal RNAs complementary to these genes. The maximum molecular weights, as well as the average molecular weights, were approximately the same in normal and CAM RNAs complementary to these genes. For genes 30 and 39 only small fractions of the radioactive RNAs made either in the presence or the absence of CAM were large enough to be full-length transcripts.

We also studied the size distribution of functional T4 mRNA in a preparation of CAM RNA. Nonradioactive RNA was isolated 10 min after infection by T4, and CAM was added 5 min before infection. The RNA was electrophoresed on a preparative RNA gel and analyzed as described in the legend to Fig. 2. No polypeptide with a molecular weight greater than about 21,000 was synthesized (41). All of the T4 CAM RNA polypeptides were synthesized by mRNA's having molecular weights of 0.1×10^5 to 0.2×10^5 (data not shown). Although no specific gene products were identified, many of the small polypeptides coded for by CAM RNAs had the same mobilities as the small polypeptides coded for by normal RNAs. However, in normal RNAs some of the small polypeptides were encoded by large mRNA's, as shown in Fig. 2, whereas in CAM RNAs no large T4 mRNA's were identified by translation of functional mRNA's.

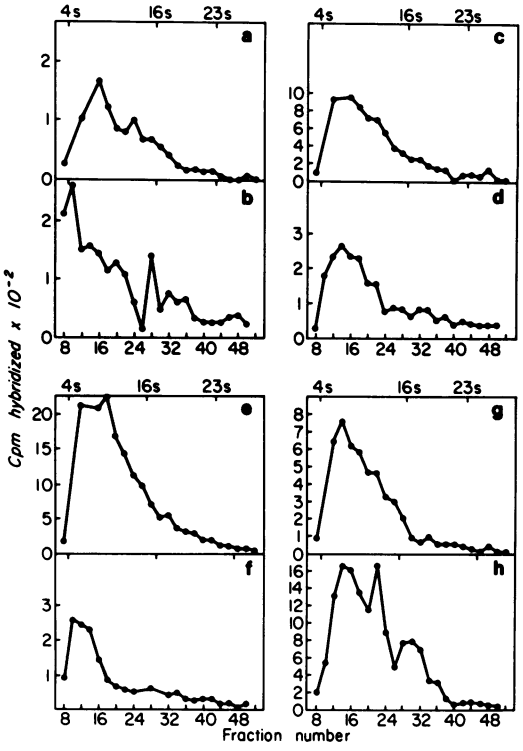

FIG. 11. *Analysis of fractionated T4 CAM-treated RNAs by hybridization to recombinant plasmids. Radioactive RNA was prepared by labeling cells with [^{3}H]uracil between 3 and 6 min after infection by T4 52amEA118 either in the absence or presence of 200 μg of CAM per ml added 5 min before infection. The RNA was isolated and analyzed by electrophoresis and hybridization as described in the legend to Fig. 9 and in the text. The RNA was denatured with glyoxal before electrophoresis as described in the legend to Fig. 10 and fractionated by electrophoresis on a 2.0% acrylamide-0.5% agarose gel; 0.9 ml of every other 1-ml fraction was hybridized to filters containing the following plasmid DNAs: (a) p625 (gene 30), no CAM; (b) p625 (gene 30), plus CAM; (c) p626A (gene 39), no CAM; (d) p626A (gene 39), plus CAM; (e) p627 (genes 40 and 41), no CAM; (f) p627 (genes 40 and 41), plus CAM; (g) p624 (genes 42 and βgt), no CAM; (h) p624 (genes 42 and βgt), plus CAM.*

DISCUSSION

With a few notable exceptions, T4 mRNA's are unstable, as are most procaryotic mRNA's. Nevertheless, estimating the molecular weights of specific T4 transcripts under different conditions, such as different times after infection, during infections with putative regulatory mutants (tsG1, regA), and during infections of mutant host cells (RNase I$^-$, RNase III$^-$), should provide information important to an understanding of the arrangement of T4 transcription units and synthesis, processing, and degradation of T4 mRNA.

Functional T4 mRNA was detected by fractionating total RNA from T4-infected cells on preparative polyacrylamide gels, followed by translation in vitro to identify specific polypeptides and, indirectly, specific mRNA's. Since we used autoradiography of one-dimensional sodium dodecyl sulfate-polyacrylamide gels to identify T4 polypeptides, only the most prominent T4 early proteins could be identified easily.

The minimum size of the mRNA detected had to be sufficient to code for the intact polypeptide chain, since that was our means of identifying the mRNA activity. Our size estimates of the T4 RNAs were based on comparisons of their mobilities (more properly, their mobilities and elution rates) with the mobilities of a series of RNAs with known molecular weights. For T7 early and late mRNA's, this method provided a fairly accurate measurement of molecular weights (26). We have no reason to believe otherwise for T4 RNAs. For four of the nine mRNA's whose sizes are shown in Table 3, we observed a single messenger peak of approximately monocistronic size. In each case the apparent molecular weight exceeded the coding capacity. Some extra nucleotides were presumably present in the flanking regions, but the apparent excess of 200 to 600 nucleotides even for those RNA species suspected to be monocistronic (such as the gene 43 message) might indicate that our size estimates were too large for all of the mRNA's. For example, the only gene 43 message activity that was detected had a molecular weight of approximately 1.2×10^6. Since gp43 has a molecular weight of 1.15×10^5, a messenger with a molecular weight of approximately 1.0×10^6 is required to code for the gp43 polypeptide. There was very little if any gene 43 mRNA activity of higher molecular weight, as might have been expected if this RNA had been derived from a larger precursor. However, rapid processing to the size observed could have prevented the identification of a large gene 43 transcript.

Several early messages displayed two or more peaks of activity. Gene 52 message activity had peaks representing RNAs with molecular weights of approximately 0.65×10^6, 1.0×10^6, and 1.3×10^6. The smallest of these could have been a monocistronic gene 52 message since gp52 has a molecular weight of about 55,000. The two larger gene 52 messages could have been polycistronic messages. The best way to determine this and to determine which other genes are linked on the putative polycistronic messages is to use plasmids containing gene 52 and adjacent regions. Only the gene 52 mRNA with a molecular weight 0.8×10^6 was observed in RNA

extracted after infection with the rII deletion mutant del1241. The deletion endpoint nearest gene 52 is very far from gene 52. This observation is interesting but has not been reproduced. It is also interesting that another deletion in this same region affects gp52 synthesis. The deletion saΔ9 (7) causes overproduction of gp52 specifically (Mattson and Bolle, personal communication). In this case the effect of the deletion on gp52 expression could be due to transcription from the rIIB promoter (Mattson, personal communication), although it may be that sequences in between rIIB and gene 52 have an effect on gene 52 transcription.

Gene 32 is represented by two active mRNA species. These have approximately the same molecular weights as two peaks of stable radioactive RNA activity that were observed after a long chase in the presence of rifampin (H. Krisch, personal communication). In this case the two peaks of radioactive RNA were associated with gp32 message activity. In both our studies and those of Krisch, most of the gp32-synthesizing activity was associated with the lower-molecular-weight gene 32 mRNA. It is also noteworthy that the RNA extracted from cells infected with a gene 32 amber mutant contained a full-sized gene 32 message.

Unlike the messages just described, several mRNA activities migrated very heterogeneously. One such mRNA coded for gp30; the minimum molecular weight of this mRNA was about 0.60×10^6, and its maximum molecular weight was 1.5×10^6 to 2.0×10^6. Several different mechanisms could give rise to mRNA's that display heterogeneous electrophoretic mobilities. They could be derived from larger precursors which are degraded randomly into fragments, some of which can still function as templates, or they could be transcription products of genetic regions lacking strong termination signals. There are also other possibilities.

Hybridization to cloned restriction fragments containing known T4 genes was performed in order to identify specific T4 mRNA's directly. Pulse-labeled RNA was used to increase the fraction of nascent and newly synthesized transcripts. For several early T4 genes a functional mRNA had been identified unambiguously by translation in vitro (genes 43, 52, 39, rIIB, and rIIA), and we had a hybridization probe to assay specifically and directly for the RNA (the gene 52 probe may have contained a small amount of non-gene 52 DNA). For genes 43, 52, 39, and rIIB the radioactive RNAs that were able to hybridize to the corresponding plasmids had size distributions that included peaks representing RNAs with the same molecular weights as the functional mRNA's detected. However, there was also a considerable amount of lower-molecular-weight RNA, presumably representing degraded mRNA. In the case of the rIIA message, essentially all of the hybridizing RNA that was detected was smaller than the functional mRNA. However, most significant was the failure to observe pulse-labeled RNA with a higher molecular weight than the molecular weight of the functional mRNA's from the same gene. It is unlikely that this failure was due to the presence of only incomplete transcripts in the pulse-labeled RNA since a 3-min pulse should have been long enough to allow synthesis of an RNA with a molecular weight of 2×10^6 to 3×10^6 (considerably larger than the transcripts detected). However, to test this hypothesis directly, RNA was labeled from 0 to 10 and from 2 to 4 min after infection with T4, the RNA was isolated immediately after labeling was terminated, and the distribution of gene 43 message was measured by hybridization to a gene 43-containing plasmid (data not shown). The RNA labeled from 0 to 10 min after infection had a distribution of gene 43-annealing RNA very similar to that shown in Fig. 9 for RNA labeled from 3 to 6 min. In the RNA pulse-labeled from 2 to 4 min after infection there was relatively more gene 43 message in the peak with a molecular weight of 1.2×10^6. Higher-molecular-weight gene 43 RNA was not detected in either RNA preparation. This suggested that the gene 43 message with a molecular weight of 1.2×10^6 represented the transcription unit for gene 43 or that processing occurred during transcription, as appears to be the case for bacteriophage T7.

Several different mechanisms could have produced the multiple transcripts from other genes, both identified (52, rIIB, 32, IPIII) and unidentified. These transcripts could have arisen by processing of a larger transcript, as is the case with the T7 early region primary transcript and several T7 late transcripts. Alternatively, they could have been synthesized from independent promoters and a common termination site. There are well-documented precedents for this possibility. Early T7 transcription by *E. coli* RNA polymerase and late T7 transcription by T7 gene 1 RNA polymerase utilize multiple promoters and a common termination signal to generate all of the early messages and several late messages, respectively (9, 11, 26). The multiple transcripts of the small DNA phages are also derived from multiple promoters and a common termination site (35). Synthesis of RNA transcripts from a single promoter and multiple termination sites could also generate multiple mRNA species. An example of such a transcription unit occurs in bacteriophage lambda. The transcripts promoted from P_l and P_r terminate

at t_l and t_r unless the lambda N protein is present. In the presence of N protein, transcription continues into more distal genes. No T4 protein with N-like activity has been identified, although anti-termination has been suggested as an explanation for the synthesis of delayed early RNA by readthrough from adjacent immediate early regions (30, 31).

The size of the RNA from the rII region is of particular interest. rIIB message activity was detected by the synthesis of either the wild-type rIIB polypeptide or the synthesis of a shorter rIIB polypeptide coded for by a mutant rIIB gene containing an internal in-phase deletion which removed approximately 300 base pairs. rIIA message activity was more difficult to detect, but was identified unambiguously by using an rIIA mutant containing an internal in-phase deletion which reduced the molecular weight of the rIIA polypeptide by about 30,000 (from 85,000 to 55,000) in our gel system. A multiple mutant containing both rII in-phase deletion mutations, a gene 32 amber mutation which produced an identifiable amber fragment, and a gene 44 amber mutation allowed us to detect and measure both rIIA mRNA and rIIB mRNA, as well as gene 32 mRNA in the same RNA preparation. Three peaks of rIIB deletion message activity were detected; these corresponded to molecular weights of approximately 0.45×10^6, 0.78×10^6, and 1.3×10^6 (designated $mrIIB_a$, $mrIIB_b$, and mrIIAB, respectively). The two smaller rIIB messages were also identified unambiguously in wild-type rII RNA (T4 32amA453 44amN82) (Fig. 2) and in RNA containing wild-type rIIA but rIIB deletion message (T4 32amH18 44amN82) (Fig. 5). The high-molecular-weight rIIB message (mrIIAB) comigrated with message activity for rIIAdelEM66. A similar comigration of rIIA and rIIB message activities was observed when we used RNA containing either wild-type rIIA and rIIB messages, rIIB delrIIA wild-type message, or rIIB wild-type rIIA deletion message. In the case of wild-type RNA, the message activities for gprIIB and for gprIIA migrated more slowly, representing higher molecular weights than when RNAs from the deletion mutants were examined. The finding that the molecular weight of the largest rIIB message was decreased by a deletion in rIIA proved that the two message activities were on the same molecule.

Hybridization to a cloned restriction fragment of rIIB confirmed the existence of two of the functional messages just described above. An rIIB probe detected peaks of rIIB RNA at molecular weights of approximately 0.5×10^6 and 0.8×10^6, approximately the same size as observed for functional rIIB message. There was also a considerable amount of lower-molecular-weight rIIB RNA, presumably representing degraded rIIB RNA. No high-molecular-weight polycistronic rIIAB RNA was detected reproducibly with either the rIIA-specific probe or the rIIB-specific probe. Essentially all of the rIIA-specific RNA was present in a heterogeneous distribution with molecular weights ranging from 2.5×10^4 to 1.0×10^6, suggesting that rIIA mRNA was broken down more rapidly than rIIB mRNA. Polycistronic rIIAB transcripts were detected by rehybridizing radioactive RNA first hybridized either to a plasmid containing the 5′-terminal end of rIIB or to a plasmid containing the 3′ terminus of rIIA.

Schmidt and co-workers (31) originally reported a polycistronic rIIAB transcript, as well as a monocistronic rIIB transcript, based on studies in which hybridization to phage DNAs containing various rII deletions was used. Our results confirm the existence of both a polycistronic rIIAB transcript and a smaller, possibly monocistronic rIIB transcript. In addition, we found a third rIIB message that was not anticipated. Other investigators have studied the size of rII RNA by using deletion hybridization techniques. Sederoff et al. (33) found rII RNA that sedimented in a broad distribution representing molecular weights ranging from 2.5×10^4 to 1.2×10^6. These authors did not distinguish between rIIA-specific and rIIB-specific sequences. This is the same molecular weight range that we observed when we used hybridization to specific rIIA and rIIB probes. On the other hand, Witmer (38) claimed that he detected an rIIB transcript with a molecular weight of 0.43×10^6, an rIIA transcript with a molecular weight of 0.71×10^6, and a polycistronic rIIAB transcript with a molecular weight of 1.1×10^6. Only the polycistronic rIIAB transcript was detected when T4 RNA synthesized in vitro was analyzed by Witmer (38). There was no lower-molecular-weight RNA present in the RNA preparations of Witmer despite repeated incubation of the RNA at an elevated temperature during hybridization before analysis of the RNA by sucrose gradient sedimentation. We cannot account for the discrepancies between our results and those of Witmer (38). In particular, we never detected a monocistronic rIIA transcript, nor have we ever examined an RNA preparation that did not contain significant amounts of lower-molecular-weight rIIA-specific RNA and rIIB-specific RNA.

It is thought that CAM inhibits synthesis of delayed early RNA by preventing elongation of transcripts from immediate early genes into delayed early genes. If this were the case, CAM-treated RNA transcribed from immediate early

genes should be smaller than normal RNA transcribed from the same genes. We tested this hypothesis by using T4 hybrid plasmids known to contain immediate early gene 30, gene 39, gene 40 plus gene 41, and gene 42 plus gene βgt (41). Only in the case of gene 40 plus gene 41 was the CAM-treated RNA significantly smaller than normal RNA. Even in this case, the CAM-treated RNAs specific for gene 40 plus gene 41 had the same range of molecular weights, but the average molecular weight was smaller. This negative evidence does not allow us to draw any conclusions regarding the validity of the hypothesis which the experiment was designed to test. There is apparently such extensive mRNA degradation in the absence and presence of CAM that the issue cannot be resolved simply by analyzing the size distribution of a single RNA species. Sederoff et al. (33) also observed that the D2 region, which is downstream from the rII genes, is transcribed into RNA of the same small size in the presence and absence of CAM. Cotranscription of adjacent immediate early and delayed early genes can now be studied in a variety of ways by using the cloned restriction fragments which are available.

The size of the gene 52 RNA synthesized in the presence of CAM was also studied (unpublished data). Gene 52 was classified as an immediate early gene based on the ability of radioactive CAM-treated RNA to anneal to a gene 52-containing plasmid (41). In subsequent experiments we found that the amount of CAM-treated RNA complementary to gene 52 is more variable than for other immediate early genes. The origin of this variability is unknown, but it might be related to the complexity of the gene 52 messages.

The observation that many T4 early messages are present in multiple size classes of RNA may be relevant to regulation of T4 early gene expression. It is commonly thought that the polycistronic rIIAB transcript is transcribed from a promoter which has properties different than the properties of the middle-mode rIIB promoter. Pribnow et al. (26a) have identified the presumed middle-mode promoter for rIIB; this promoter lies withi the rIIA gene. Whether this promoter is used to transcribe both small rIIB trancripts ($mrIIB_a$ and $mrIIB_b$) is unknown. If it does, our results suggest that the termination site for the $mrIIB_a$ transcript is not 100% efficient and that readthrough to a second termination site about 1,000 nucleotides downstream from the first termination site produces $mrIIB_b$. Alternatively, there could be a second promoter within rIIA. We prefer the first alternative beause like rIIB, the D1 region adjacent to rIIB is a delayed early region, and it is approximately 700 base pairs long (7).

An alternative location for the termination of transcription for mrIIBa (the smallest rIIB transcript) is at the end of the D1 region rather than at the end of rIIB. A termination site between D1 and D2 was proposed by Sederoff et al. (33) to explain the difference between the distributions of rII and D1 RNAs and the distribution of D2 RNA on sucrose gradients. Since our estimate of the size of $mrIIB_a$ indicated that it has about 750 base pairs more than needed to encode rIIB, this transcript might contain the D1 region, which is about 700 base pairs long (7). This interpretation suggests that some genes in the D2 region (denB, pla262, ndd, stp, and ac) (7), some of which should be immediate early genes according to hybridization data (33), are cotranscribed with rIIB and D1 to produce polycistronic $mrIIB_b$ mRNA. At least some of the D2 region transcripts are synthesized in the presence of CAM (32, 33). We predict that these transcripts are from the D2b region, which is adjacent to gene 52 (32). Sederoff et al. (R. Sederoff, A. Bolle, and R. H. Epstein, unpublished data) have suggested that there are three different promoters for D1, D2a, and D2b. We suggest instead that D1 and D2a are synthesized from the same rIIB middle-mode promoter but that they are on different transcripts by virtue of anti-termination at a site between D1 and D2a. The D2b promoter in this model is an immediate early promoter, and its transcript could include gene 52.

The multiple mRNA species that represent, for example, genes 32, 52, and IPIII, could also be indicative of multiple promoters, as in the case of rIIB. Whether or not this is the case, the multiplicity of mRNA species that represent a single gene suggests that there could be different modes for transcribing the same gene under the influence of different regulatory factors. These observations could explain, for example, the failure to find mutants that are pleiotropically negative for the synthesis of one class of RNA. If most of the T4 early genes could be transcribed from more than one promoter and the promoters were in different regulatory classes, then no defect in a single regulatory gene would be sufficient to prevent expression completely. Pulitzer et al. have developed this idea more fully (27). One case may involve mutations in the *mot* gene, which were first identified by Mattson et al. (18). *mot* mutations are characterized by a decrease in the expression of several early genes (including 32, rIIB, 43, 45, 46, and tRNA) and an extended period of expression of others (including 52, rIIA, and 44) (20, 27). These effects are probably transcriptional (18, 27). Based on UV inactivation of gp43-synthesizing ability, Her-

cules and Sauerbier (15) suggested that gene 43 is transcribed initially from a distant promoter and subsequently from an adjacent promoter and that the latter is not utilized during infection by a *mot* mutant. Several of the *mot*-affected genes are represented by two or three different messages (genes 32, rIIB, 46, 52, and probably 45) but others are not (genes rIIA and 43). Gene 43 could be an exception, or we might have failed to detect a large transcript because of instability or processing. The effect of the *mot* mutation on the size distribution of these specific RNAs should reveal whether the *mot* mutation affects one or all transcripts from genes 32, rIIB, 52, and 45.

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