Destabilization of Bacteriophage T4 mRNAs by a Mutation of Gene 61.5

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ABSTRACT

We identified a novel gene of bacteriophage T4, gene 61.5, which appears to be involved in protein synthesis late in infection. Northern blot analysis revealed that a mutant of 61.5 accumulated truncated transcripts of representative late genes. Using a double mutant of genes 61.5 and 55, which prevents transcription of late genes, we demonstrate that even transcripts of middle genes, while full-length when initially expressed, are similarly truncated at later stages of infection. These results indicate that the abnormality in transcript length occurs late in infection, regardless of whether the transcript derives from a middle or a late gene. Primer-extension analysis revealed that the 5' ends of the late gene 23 transcripts that accumulated in gene 61.5 mutant-infected cells were located at internal discrete sites as well as at the expected transcription start site. Moreover, the decay rates of full-length transcripts from genes *uvrY* or 45 were more than twofold faster in the absence of a functional gene 61.5. These results suggest that mutation of gene 61.5 activates endonucleolytic cleavage of middle and late transcripts, probably by RNase M.

IN *Escherichia coli* cells infected with bacteriophage T4, gene expression is tightly regulated. Different sets of proteins, early, middle and late, are sequentially synthesized as the infection cycle proceeds. Such highly ordered gene expression is accomplished via the coordination of many different T4 genes whose products regulate the sequential modification of RNA polymerase to recognize different classes of promoters, processing and degrading mRNAs to allow rapid transitions in the mRNA pool, and translational repression to prevent inappropriate protein synthesis (MOSIG and HALL 1994). The extensive genetic analyses of bacteriophage T4 conducted so far have identified nearly 200 genes. However, there are many more protein spots in two-dimensional polyacrylamide gel analyses than can be explained by known genes (KUTTER *et al.* 1994a). This difference is consistent with the >100 ORFs (open reading frames) in the T4 genome (KUTTER *et al.* 1994b). These observations suggest that there remain genes to be identified that are involved in the regulation of gene expression.

The intergenic region between gene 41 (replicative DNA helicase) and gene 61 (DNA primase) contains five small ORFs, 61.1–61.5 (SELICK *et al.* 1993). The predicted proteins range in size from 6 to 23 kDa and bear no discernible sequence homology with known proteins. ORFs 61.1, 61.3, 61.4 and 61.5 are highly conserved in two closely related phages, T2 and T6. ORF 61.2 is highly conserved in T6 but is replaced by an unrelated ORF in T2. That these ORFs are indeed genes

is supported by their codon preferences, which are indistinguishable from those of known T4 genes. In the region surrounding them on the T4 chromosome are located genes for DNA replication, DNA recombination, and the regulation of gene expression. Because there is a strong tendency for genes that encode functionally related proteins to be clustered on the T4 chromosome, ORFs 61.1–61.5 are expected to be involved in DNA replication, DNA recombination, or the regulation of gene expression.

We report here that T4 bearing an amber mutant of gene 61.5 propagates poorly on nonsuppressing *E. coli* K-12 hosts at low temperatures. Under the restrictive conditions, the level of protein synthesis is dramatically reduced at late stages of infection. Analysis of the transcripts that accumulate in the mutant-infected cells strongly suggests that T4 mRNAs are destabilized at late stages of infection by a mutation of gene 61.5.

MATERIALS AND METHODS

Phage and bacterial strains: The wild-type and mutant phages were T4D, *amSF16* (gene 61.5) and *amBL292* (gene 55). *E. coli* K-12 strains were MH1 (*sup^h* *araD139* Δ *lacX74* *galU⁻* *galK⁻* *hsr⁻* *rpsL⁻*), 594 (*sup⁰*), K38 (*sup^h*) and CR63 (*sup^D*).

Construction of an amber mutant of gene 61.5: A 145-bp DNA fragment obtained from p415 (YONESAKI 1994) by cleavage with restriction enzymes *MnII* and *HindIII* contained the sequence encoding the amino-terminal 46 amino acids of gene 61.5. This fragment was cloned into the I/S (insertion/substitution) vector pBSLO+, which encodes a *supF* tRNA (SELICK *et al.* 1988). The resulting plasmid was further inserted with the DNA replication origin of phage fd to yield the plasmid pTAR 61.5. Circular single-stranded DNA of

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pTAR 61.5 was prepared by infecting *E. coli* JM101 cells harboring this plasmid with VCSM13 helper phage.

The mutagenic oligonucleotide 5'-GTTTAATAATGAATAGATGTTTACTAAG was complementary to the sequence extending from nucleotides 33 to 61 of the gene-61.5 coding region, with the exception of the middle three nucleotides, TAG. These nucleotides were introduced to convert the original serine codon at the position of amino acid 16 to an amber termination codon. The mutagenic oligonucleotide was annealed to pTAR 61.5 single-stranded DNA and used as a primer for DNA synthesis by T4 DNA polymerase. The resulting open circular double-stranded DNA was then sealed with T4 DNA ligase and used to transform *E. coli* MH1. Plasmid DNA was prepared from a mixture of transformants and used for a successive transformation. Clones were then isolated by colony hybridization using the mutagenic oligonucleotide as a probe.

The mutant sequence in the plasmid was then substituted for its wild-type counterpart in the T4 chromosome by the insertion/substitution method (SELICK *et al.* 1988). Briefly, a clone was grown in liquid culture and infected with T4 I/S phage. The I/S phage possesses amber mutations in two essential genes and cannot propagate on nonsuppressing MH1 cells. Upon integration of the pBSLO+ vector via homologous recombination with T4 sequences, expression of the plasmid *supF* tRNA supports the propagation of I/S phage. Integrants are therefore selected by their ability to grow on nonsuppressing MH1 cells. After one cycle of propagation, segregants that have lost the vector DNA sequences by intrachromosomal recombination are identified by their inability to grow on MH1 cells compared to the suppressing host CR63. Because segregants could possess either a wild-type or mutant gene 61.5, they were screened for the mutant gene by plaque hybridization using the mutagenic oligonucleotide as a probe. The genetic background of the I/S phage was removed by backcrossing with wild-type phage. The presence of the desired gene-61.5 mutation in the resulting *amSF16* strain was confirmed by DNA sequence analysis using AMV reverse transcriptase.

RNA purification: RNAs were purified by the method described by YOUNG *et al.* (1980) with modifications. Cells grown to 3×10^8 cells/ml in M9A (YONESAKI and MINAGAWA 1987) were infected with T4 bacteriophage at a m.o.i. (multiplicity of infection) of 10. At various times after infection, 10-ml aliquots were quickly chilled on ice and centrifuged at $8000 \times g$ for 10 min at 4°, and the cells were resuspended in 200 µl of an ice-cold buffer consisting of 0.15 M NaCl, 10 mM Tris·Cl (pH 6.8) and 1 mM EDTA (pH 8.0). After adding 50 µl of 0.25 M Tris·Cl (pH 6.8), 10 mM EDTA (pH 8.0) and 5% SDS (sodium dodecyl sulfate), the mixture was incubated at 65° for 3 min. Sodium acetate (pH 5.2) was added to a final concentration of 0.2 M, and nucleic acids were extracted with phenol/chloroform previously equilibrated with 0.2 M sodium acetate (pH 5.2) and then precipitated by adding ethanol and collected by centrifugation. The nucleic acids were dissolved in 200 µl of 40 mM Tris·HCl (pH 7.9), 10 mM NaCl, 6 mM MgCl₂ and 0.1 mM CaCl₂, and treated with 2 units of RNase-free DNase (Epicentre) at 37° for 10 min. Following the removal of DNase by phenol/chloroform extraction, RNA was collected by ethanol precipitation.

Northern blot analysis: RNAs were denatured in the presence of 6% formaldehyde and 50% formamide at 65° for 5 min, and electrophoresed through a 1.2% agarose gel containing 6% formaldehyde, 20 mM N-morpholinopropanesulfonic acid (pH 7.0), 50 mM sodium acetate, and 10 mM EDTA (pH 8.0). The integrity and quantity of RNA in each lane were monitored by visualizing the rRNAs upon staining with ethidium bromide. RNAs were transferred to a nylon mem-

brane and cross-linked by UV irradiation. The membrane was hybridized with ³²P-labeled DNA probe at 42° overnight in 50% formamide, 0.25 M sodium phosphate (pH 7.0), 0.2 mM EDTA (pH 8.0), 0.25 M sodium chloride and 3.5% SDS. The membrane was washed at room temperature with 5× SSC containing 0.1% SDS and then washed at 42° in 1× SSC containing 0.1% SDS.

Radioactively labeled probes for gene *uvrY* were prepared by either nick-translating or end-labeling the 0.8-kb fragment obtained from pBSK1 (YONESAKI and MINAGAWA 1987) after digestion with restriction enzymes *Clal* and *Bgl*II. All other probes were prepared by labeling via PCR. Before amplification, the primer that was complementary to the desired mRNA was end-labeled using T4 kinase and [γ -³²P]ATP. The primer sequences that were used are as follows: for gene 23, 5'-CCGAACGAAATGGAT and 5'-³²P-CCCTAAAATTGTGT; for gene 37, 5'-TGCGCGGGCAAATA and 5'-³²P-AGCACT-GCTAGTCCC; for gene 45, 5'-CCCGGTAGCATCTGC and 5'-³²P-GTGGGTAGAATCAGC; for gene 51, 5'-CACTGATAT-AGTAGA and 5'-³²P-CGGCCATAAAGAGAA.

Sequencing and primer extension: RNA sequencing and primer extension were carried out as described by MCPHEETERS *et al.* (1986) with some modifications. The DNA primer, 5'-CTGACCTGCAGCGATGTTGGTAGC, was complementary to the sequence located 380 nucleotides (nt) downstream of the initiation site of the gene-23 monocistronic transcript (KASSAVETIS and GEIDUSCHEK 1982). One pmol of the DNA primer was labeled with ³²P at the 5' end and mixed with 50 µg of purified RNA in 12 µl of 50 mM Tris·Cl (pH 8.3), 60 mM NaCl and 10 mM dithiothreitol (DTT). After incubating for 3 min at 60°, the mixture was quickly chilled on ice. Then, 1.4 units of AMV reverse transcriptase was added to 2 µl of the mixture that was then brought to 5 µl by the addition of 50 mM Tris·HCl (pH 8.3), 60 mM NaCl, 10 mM DTT, 6 mM Mg(OAc)₂, and 0.4 mM of each dNTP. For sequencing, one of each ddNTP (0.25 mM ddATP, 0.15 mM ddGTP, 0.15 mM ddCTP and 0.1 mM ddTTP) was also included. The primer was extended at 37° for 3 min, and then at 54° for an additional 20 min. The reaction was stopped by the addition of 10 µl of 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue, 10 mM EDTA (pH 8.0) and 95% (v/v) deionized formaldehyde. The reaction products were denatured by boiling for 2 min and analyzed by gel electrophoresis.

RESULTS

An amber mutant of gene 61.5: Using the T4 insertion/substitution vector system, we constructed an amber mutant of gene 61.5 (see MATERIALS AND METHODS). The mutant, designated *amSF16*, was defective in propagation on the *E. coli* K-12 nonsuppressing host, MH1, in a temperature-dependent manner. The ratio of efficiency of plating on MH1 over that on the suppressing host, CR63, at 42°, 37°, and 32° was 0.6, 0.2, and <0.01, respectively. In comparison with the wild type, the plaque size of the mutant was nearly normal at 42°, small at 37°, and tiny at 32°. The mutant was similarly defective on other nonsuppressing strains (594 and K38) at low temperatures.

To address the cause of the propagation defect, we first examined DNA synthesis by monitoring the incorporation of [³H]thymidine into the TCA (trichloroacetic acid)-precipitable fraction. The result was the same as that of the wild type (data not shown), indicating

FIGURE 1.—Agarose gel electrophoresis of total intracellular *E. coli* DNA following T4 infection. MH1 cells were grown in M9A (YONESAKI and MINAGAWA 1987) to 5×10^8 cells/ml and infected at 30° with T4D or *amSF16* at a m.o.i. of 5 (time 0). At the times indicated above the figure, the infected cells from 0.5-ml aliquot were collected by centrifugation and resuspended in 0.1 ml of 100 mM NaCl, 50 mM Tris·Cl (pH 7.8), 10 mM EDTA (pH 8.0), 0.2% SDS and 500 μ g/ml proteinase K. After incubation for 2 hr at 37°, the lysate was denatured in 0.3 M NaOH and electrophoresed at 20 V for 20 hr through 0.25% agarose gels containing 1 M urea in a buffer consisting of 40 mM Tris-acetate and 1 mM EDTA (YONESAKI 1994). DNAs were visualized by staining with ethidium bromide. The leftmost lane contained DNA markers, T4 phage DNA (171 kb) and λ phage DNA (48.5 kb).

that DNA synthesis by the mutant is normal. Because a defect in DNA recombination often accompanies impairment of DNA synthesis in T4 metabolism (YONESAKI 1995), this result suggested that DNA recombination may be also normal. This conclusion was strengthened by the observations that the mutant was as sensitive to UV irradiation as the wild type (data not shown), indicating that it retains recombination-mediated repair, and that concatemeric DNA (which is the consequence of DNA recombination) was produced abundantly in the mutant-infected cells, as described below.

Intracellular T4 DNA was extracted at various times after infection at 30° and analyzed by agarose gel electrophoresis (Figure 1). In wild-type-infected cells, formation of concatemeric DNA and maturation of DNA into single chromosome equivalents were detectable at 20 min. Accumulation of mature DNA progressed through 40 min. In mutant-infected cells, concatemeric DNA also appeared at 20 min in quantities comparable to that seen in the wild-type-infected cells. However, maturation of DNA was undetectable through 90 min, with concatemeric DNA accumulating instead. Because the maturation of T4 DNA occurs by the cleavage of concatemeric DNA after packaging into a capsid, this

FIGURE 2.—Polyacrylamide gel electrophoresis of T4 proteins. MH1 cells were grown and infected with T4 phage as described in Figure 1. Infected cells were pulse-labeled for 2 min with [35 S]methionine and [35 S]cysteine at 200 μ Ci/ml at the times indicated above the figure. Proteins were extracted from infected cells and electrophoresed through a 10% polyacrylamide gel containing 0.1% SDS. An autoradiograph was taken with a Bio-Image Analyzer (Fuji BAS-2000II) by exposing the gel to an imaging plate. Arrows indicate early and middle proteins, and arrowheads indicate late proteins.

observation suggests that either packaging or cleavage of concatemers is blocked by the gene 61.5 mutation.

Late protein synthesis by the mutant: To further understand the defect in DNA maturation, we investigated protein synthesis in the *amSF16* mutant. Newly synthesized proteins were pulse-labeled with [35 S]methionine and [35 S]cysteine for 2 min at various times after infection at 30° and analyzed by polyacrylamide gel electrophoresis (Figure 2). In the wild-type-infected cells, different classes of proteins synthesized sequentially were discernible: early (3–5 min), middle (8–10 min) and late proteins (18–20 and 28–30 min). In the *amSF16*-infected cells, late proteins were hardly synthesized, whereas early and middle proteins were synthesized as timely and efficiently as those in the wild-type-infected cells. Thus, *amSF16* is defective in the synthesis of late

FIGURE 3.—Northern blotting of T4 transcripts. Each lane contained 5 μ g of total RNA extracted from the T4D- or *amSF16*-infected cells at 4, 11, 19 and 29 min after infection at 30°. RNAs were electrophoresed, transferred to a nylon membrane, and hybridized to 32 P-labeled DNA probes representative of typical late and middle genes, as described in MATERIALS AND METHODS. The positions of rRNAs (2.9 and 1.5 kb) are indicated by dots at the right margin. The probe used was for gene 23 (A), gene 37 (B), gene 51 (C), gene 45 (D) and *uvsY* gene (E). Arrowheads indicate full-length transcripts of each gene. An arrow in E indicates the 2.0-kb late transcript (see text).

proteins, a result consistent with the defect in DNA maturation (Figure 1) (because late proteins include those required for DNA packaging and cleavage).

The absence of full-length transcripts of late genes: To investigate the reason for the poor synthesis of late proteins, we examined RNA synthesis by the incorporation of [3 H]uridine into the TCA-insoluble fraction. The result showed no significant difference from that of the wild type throughout infection (data not shown), suggesting that on a quantitative basis, the overall transcriptional activity of the *amSF16* mutant is normal. However, when analyzed by Northern blotting, it became apparent that the full-length transcripts of three late genes that we tested were absent in the *amSF16*-infected cells. The arrowheads in Figure 3, A–C denote monocistronic (1.8 kb) and polycistronic (2.5 and 3.5 kb) transcripts of gene 23 (YOUNG *et al.* 1981; KASSAVETIS and GEIDUSCHEK 1982), polycistronic transcripts (4–5 kb) of gene 37, and polycistronic transcript (4 kb) of gene 51. These transcripts were detected as discrete bands via Northern analysis after 11 min of infection by wild-type bacteriophage. In contrast, they were not observed in the RNAs isolated from *amSF16*-infected cells. Instead, only short RNA species accumulated. This observation might be biased by the use of DNA probes

that were complementary to the 3'-terminal region of each gene. However, a comparable result was obtained with the gene 23 transcript using a probe complementary to the 5'-terminal region of that gene (data not shown). Gene 23 encodes a major T4 head protein comprising 521 amino acids. Nevertheless, the gene 23 transcripts found in the mutant-infected cells were shorter than 1.5 kb, indicating that these transcripts cannot be functional as a message of that gene. These results suggest that the global defect in late protein synthesis is due to the absence of full-length transcripts of late T4 genes in *amSF16*-infected cells.

The transcripts of middle genes were similarly examined. In wild-type-infected cells, full-length polycistronic (4 kb) and monocistronic (0.9 kb) transcripts of gene 45 and monocistronic transcript of *uvsY* (1.2 kb) were observed at 4 and 11 min (Figure 3, D and E). A 2.0-kb transcript was also detected with the *uvsY* probe at 11, 19 and 29 min in wild-type-infected cells, consistent with this gene also being transcribed late in infection (GRUIDL *et al.* 1991). In *amSF16*-infected cells, the same transcripts were also observed at 4 min. However, the polycistronic transcript of gene 45 was less abundant than following wild-type infection and the monocistronic transcript was reduced to heterogeneously

FIGURE 4.—T4 proteins synthesized in the absence of gene 55 protein. Proteins synthesized in *amBL292*- or *amBL292 amSF16*-infected cells were labeled with [³⁵S]methionine and [³⁵S]cysteine and analyzed by polyacrylamide gel electrophoresis as described in Figure 2.

shorter sizes at 11 min, as were the monocistronic transcripts of the middle- and late-expressed *uvrY* gene. The reduction in the length of the transcripts was therefore shared by representative late as well as middle genes in *amSF16*-infected cells.

The absence of full-length transcripts at late stages of infection: To further examine the effect of the *amSF16* mutation on the length of transcripts late in infection, we constructed a double mutant of genes 55 and 61.5 by a standard cross between *amBL292* and *amSF16*. Because gene 55 encodes the σ factor required for late promoter recognition (KASSAVETIS and GEIDUSCHEK 1984; MALIK and GOLDFARB 1988), a 55⁻ mutant cannot transcribe late genes efficiently and instead persists in the transcription of middle genes. As expected, the gene 55 mutant was virtually unable to synthesize late proteins, and most proteins found at late stages were middle proteins (Figure 4; compare with wild type in Figure 2). In fact, the full-length (1.2-kb) monocistronic transcript of the middle gene *uvrY* was observed until

FIGURE 5.—Prolonged transcription of the middle gene *uvrY*. After MH1 cells were infected with *amBL292* or *amBL292 amSF16*, total RNAs were extracted from the cells at the times indicated above the figure and were analyzed as described in Figure 3.

late stages following infection by this mutant (Figure 5). In the double-mutant-infected cells, however, early and middle proteins were synthesized normally until the middle stages of infection, but no protein was synthesized at late stages (Figure 4). Consistently, the 1.2-kb transcript was observed until middle stages, but short species became prominent in place of the full-length transcript at later stages (Figure 5). These results suggest that the gene 61.5 mutation eliminates the full-length functional transcripts of middle as well as late genes at late stages of infection.

Destabilization of mRNA: Elimination of the full-length transcripts could be explained by increased degradation of RNA. To explore this possibility, we measured the decay rate of full-length transcripts in the presence or absence of a functional gene 61.5. Rifampicin was added at 7 min after infection to inhibit further transcription, and total RNAs were then extracted from the cells at 2-min intervals. The amount of the full-length *uvrY* transcript at each time was quantified after Northern blotting (Figure 6A), and its relative amount was plotted to determine the half-life of the transcript (Figure 6B). The full-length transcript had a $t_{1/2}$ of 2.4 min in *amBL292 amSF16*-infected cells, and of 5.8 min in *amBL292*-infected cells. Thus, the *uvrY* gene transcript was degraded 2.5-fold faster in the absence of functional gene 61.5 than that in its presence. Under the same conditions, the full-length mono- and polycistronic transcripts of gene 45 were also degraded more than twofold faster (data not shown) than following infection by wild-type T4. Therefore, mutation of gene 61.5 causes a rapid decay of mRNAs.

Uniquely processed 5' ends of gene 23 transcripts: By characterizing their terminal structures, we found that transcripts of the late gene 23 in *amSF16*-infected

FIGURE 6.—Decay of the full-length transcript of gene *uvsY*. At 7 min after MH1 cells were infected with *amBL292* or *amBL292 amSF16* as described in Figure 1, rifampicin was added (time 0) to 200 $\mu\text{g}/\text{ml}$ to inhibit further initiation of transcription. (A) Total RNA was prepared from the cells every 2 min as indicated above the figure and the 1.2-kb *uvsY* transcript was detected by Northern blotting as described in Figure 3E. (B) The amount of 1.2-kb *uvsY* transcript in each lane was quantified using a Bio-Image Analyzer (Fuji BAS-2000II) and expressed as the percentage of that at 2 min. Results are means $\pm$ SD of three independent experiments.

cells had 5' ends at unique discrete sites as well as at transcription start site. For this analysis, cDNAs were produced from gene 23 transcripts by primer extension using AMV reverse transcriptase and were resolved by electrophoresis through a polyacrylamide gel (Figure 7A). When RNA from either wild-type- or mutant-infected cells was used as a template, a 380-nt cDNA fragment (indicated by an arrow in the figure) was the most prominent. This corresponds to the 5' moiety of monocistronic transcript that was initiated immediately upstream of the gene 23 coding region (KASSAVETIS and GEIDUSCHEK 1982). Other cDNAs longer and shorter than the 380-nt cDNA were also shared in the

wild-type- and mutant-infected cells. They may result from premature termination of cDNA synthesis from polycistronic and monocistronic templates or from truncation of transcripts at discrete sites via a common mechanism of mRNA degradation. However, several cDNAs were observed (shown by arrowheads) only with RNA prepared following *amSF16* infection. Because these unique cDNAs cannot be due to premature termination by AMV reverse transcriptase, they should represent the 5' ends of transcripts of gene 23 that were cleaved at discrete internal sites and that accumulated in the mutant-infected cells.

To deduce the 5'-terminal sequences of these truncated fragments, the cDNAs were electrophoresed through a standard sequencing gel along with a DNA sequencing ladder produced by dideoxy sequencing using the same primer and wild-type RNA as a template (data not shown). From that result, we assigned 11 unique cDNAs derived from the mutant RNA and deduced all of the 5' ends of the corresponding transcripts (Figure 7B, capital letters indicating the terminal nucleotides at 5' ends). All 11 sites were characterized by a pyrimidine nucleotide upstream of the cleavage site, and 10 of the 11 5' terminal nucleotides were purines. These results strongly suggest that the 5' termini are generated by endoribonucleolytic cleavage of the transcript at multiple discrete sites (see DISCUSSION).

DISCUSSION

In the present study, we report the construction of a strain of bacteriophage T4 with an amber mutation in gene 61.5. This mutant is defective in protein synthesis at late stages of infection at reduced temperatures, indicating that gene 61.5 plays an important role in gene expression. As judged by the incorporation of labeled nucleotide precursor, net RNA synthesis by the mutant is normal. However, transcripts of full length are absent and shorter species accumulate at late stages of infection. This is presumably why protein synthesis is defective, as exemplified by the truncation of the gene-23 transcript to an extent that will no longer support the synthesis of functional coat protein.

The absence of full-length transcripts and accumulation of short species might be explained by premature termination of transcription or abnormal initiation at sites other than promoters. Premature termination is unlikely, however, because short transcripts of gene 23 are detected by hybridization with DNA probes complementary to the 3' terminal sequence of the coding region (Figure 3A). Abnormal initiation also seems unlikely for several reasons. T4 middle and late genes are transcribed by two different forms of RNA polymerase (WILLIAMS *et al.* 1994). Furthermore, the transcription of middle genes requires the participation of a transcriptional factor, the *motA* protein (OUHAMMOUCH *et al.* 1995), while transcription of late genes requires

FIGURE 7.—Identification of the 5' ends of gene 23 transcripts. RNA was prepared from T4D- or *amSF16*-infected cells at 20 min after infection at 30° and used as a template for primer extension as described in MATERIALS AND METHODS. (A) The cDNA products were denatured by boiling for 2 min and electrophoresed through a 4% polyacrylamide gel containing 7 M urea. The left lane contained the size markers. The arrow indicates the product derived from the full-length monocistronic transcript. The arrowheads indicate cDNAs produced only from the mutant RNA. (B) The 5' and its upstream sequences of the gene 23 coding region is presented. Large letters indicate the nucleotides at 5' ends detected only with mutant RNA. Asterisks mark dinucleotides consisting of the 5'-terminal nucleotides and their adjacent upstream nucleotides.

DNA replication as an enhancer (TINKER *et al.* 1994). Despite these differences in transcription initiation mechanisms, the reduction in transcript length is found with both classes of genes. However, endoribonucleolytic cleavage of mRNAs, as observed with those transcribed from gene 23, can explain the absence of full-length transcripts at later stages of infection by the mutant. This is also consistent with the shortened half-life of the *uvrY* and 45 transcripts following infection by the mutant.

The results of the primer extension analyses suggest that the putative RNase cleaves RNA within the dinucleotide 5'-YR (Y = C or U, R = A or G) and 5'-CU (Figure 7B). Two well characterized endoribonucleases cleave T4 mRNAs: the host-encoded RNase E (MUDD *et al.* 1990) and the T4-encoded regB protein (SANSON and UZAN 1993). They show stringent sequence specificities, with an A+U-rich region and a higher-order structure comprising the recognition sequence for RNase E (MCDOWALL *et al.* 1994) and 5'-GGAG the recognition sequence for regB (SANSON and UZAN 1993). Because of these specificities, it is unlikely that these endoribo-

nucleases contribute to the rapid degradation of mRNAs observed in the mutant-infected cells. Alternately, among the endoribonucleases identified so far in *E. coli* and T4 systems, host-encoded RNase M may be the best candidate. RNase M has a preference for hydrolysis of 5'-YN bonds (N = any nucleotide) and, like pancreatic RNase A, the preference order is YA > YG > YY (CANNISTRARO and KENNEL 1989). This enzyme is presumed to be a primary endoribonuclease for mRNA degradation in *E. coli* (CANNISTRARO and KENNEL 1993). Its cleavage site preferences are consistent with those reported in the present study for the gene 23 transcripts, although this by no means rules out the involvement of other nucleases in this process.

At present, it is not clear whether the product of gene 61.5 inhibits the RNase M-like endoribonuclease directly or indirectly, nor whether the protein stabilizes mRNAs directly or indirectly. In *E. coli* cells, the reduced efficiency of translation initiation destabilizes mRNAs (YARCHUK *et al.* 1992). Therefore, increased degradation of mRNAs late in infection by the 61.5 mutant could be a consequence of the impairment of protein

synthesis rather than its cause. If this is the case, the gene *61.5* protein should be required for translation late in infection.

Finally, consistent with the cold-sensitive phenotype, we have found that transcripts of gene *23* are normal in length at high temperatures (data not shown). Accordingly, the RNase M-like endoribonuclease activity is enhanced by the gene *61.5* mutation only at low temperatures. This suggests several possibilities: the nuclease is heat-labile, the nuclease gene expresses only at low temperatures, a factor other than gene *61.5* protein is active at high temperatures to suppress the nuclease activity, or gene *61.5* protein is required for translation only at low temperatures. Characterization of the endoribonuclease and/or the translational machine will clarify these possibilities as well as help to elucidate the function of the gene *61.5* product in protein synthesis.

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