Cloning and Expression of Gene 4 of Bacteriophage T7 and Creation and Analysis of T7 Mutants Lacking the 4A Primase/Helicase or the 4B Helicase

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T7 gene 4, which is required for DNA replication, specifies two proteins whose coding sequences overlap in the same reading frame: the 4A protein, a 566-amino acid primase/helicase, and the 4B protein, a 503-amino acid helicase whose initiation codon is the 64th codon of the 4A protein. To study better the individual functions of these two overlapping proteins, we made clones that express both 4A and 4B proteins, only 4B protein, or only that which we refer to as the 4A' protein, in which methionine 64 is replaced by leucine, thereby eliminating the 4B initiation codon. These clones provide considerably more gene 4 protein for biochemical analysis than do infected cells. They can also be used to isolate and propagate T7 gene 4 deletion mutants, and we have made T7 mutants which lack all gene 4 coding sequences, or which express 4A' protein but no 4B protein, or 4B protein but no 4A protein. Analysis of these phage mutants shows that 4A' protein without any 4B protein can support essentially normal replication and growth, whereas 4B protein without any 4A protein supports little replication or growth. Apparently, the primase activity of the 4A protein is essential for replication, but the 4B protein is dispensable, presumably because the 4A protein also supplies helicase activity. The mutation at amino acid 64 of 4A' appears to have little effect on 4A function. The rate of replication during normal T7 infection appears to be limited by the amount of gene 4 protein, but too high a level of either 4A or 4B protein is inhibitory to growth.

T7 gene 4 is required for replication (Studier, 1969), and it specifies two proteins that are made in approximately equal amounts (Studier, 1972; Dunn and Studier, 1981). The nucleotide sequence indicates that the two proteins are translated in the same reading frame from separate initiation codons and a common termination codon (Dunn and Studier, 1983). The two gene 4 proteins co-purify from infected cells and have primase and DNA helicase activities that are needed for replication (reviewed in Richardson et al., 1987). The 503-amino acid 4B protein, expressed and purified from the cloned coding sequence, has helicase but not primase activity (Bernstein and Richardson, 1988). The 566-amino acid 4A protein was not obtained free of 4B, but the behavior of a mixture enriched for 4A suggests that the 4A protein itself may have both primase and helicase activity (Bernstein and Richardson, 1989).

To understand better the role of the 4A and 4B proteins during T7 infection and their possible biochemical functions and interactions, it is useful to be able to express the two proteins individually or together from the cloned coding sequences. Not only should clones provide a better source of protein than infected cells, but active protein from a clone should allow T7 mutants deficient in 4A or 4B to be propagated, thereby allowing a genetic dissection of the role of the two proteins in T7 infection.

A difficulty in this approach is the toxicity of gene 4 clones to Escherichia coli: clones that can express 4B are quite toxic (Studier, 1991), and our initial attempts to obtain clones that express both 4A and 4B were unsuccessful. Clones have been used by others to produce the 4B and mixed 4AB proteins (Bernstein and Richardson, 1988; Nekai and Richardson, 1988) but have not been described. We also wanted to express 4A alone, which requires some means of preventing the normally associated synthesis of 4B. In this paper we describe solutions to these problems, and we analyze the effect of loss of either 4A or 4B protein on T7 replication and growth. Purification and biochemical analysis of the proteins expressed from clones are described in the accompanying paper (Patel et al., 1992).

MATERIALS AND METHODS

Bacteria, Phage Strains, Plasmids, and Expression System—E. coli strains BL21, HMS174, C1757, 011' and R594 have been described (Studier, 1975; Studier and Moffatt, 1986), as have wild-type and amber mutants of bacteriophage T7 and methods for working with them (Studier, 1969). The entire nucleotide sequence of T7 DNA and locations of its genetic elements are given by Dunn and Studier (1983).

The pET vectors are derivatives of pBR322 and contain a promoter that directs transcription by T7 RNA polymerase counterclockwise across the BamHI cloning site (Rosenberg et al., 1987; Dubendorff and Studier, 1991) (refer to Fig. 1 for orientations of various elements in the plasmids): pET-1 contains the strong ϕ10 promoter; pET-10 contains the T7lac promoter, which is tightly controlled by lac repressor supplied by the lacI gene in the vector. Genes under control of a T7 promoter, as in a pET vector, can be transcribed by the T7 RNA polymerase made during T7 infection, thereby complementing defects in the phage (Campbell et al., 1978; Studier and Rosenberg, 1983).
1981. They can also be induced by IPTG in hosts such as BL21(DE3), HMS174(DE3), or C1757(D3E), which contain a chromosomal copy of the gene for T7 RNA polymerase under control of the lacUV5 promoter (Studier and Moffatt, 1986; Studier et al., 1990). The anti-sense cloning vector pBR322(A1), isolation number pAR960, carries a 286-bp fragment of T7 DNA (bp 258-543) containing the strong A1 promoter for E. coli RNA polymerase, which directs transcription clockwise across the BamHI site (for further details available from A. H. R.).

Gene 4 Clones—The gene 4 clones used in this work were constructed by standard techniques (Studier and Rosenberg, 1981; Maniatis et al., 1982). All of the clones and vectors are based ultimately on pBR322, and genes were typically cloned in the counterclockwise orientation in the BamHI site by means of BamHI linkers. Cleavage at the unique PstI site in the bla gene, and partial BamHI cleavage was a convenient way to obtain fragments from clones and vectors that could be joined to create homologous plasmids with changes in promoter composition (see Table II). Enzymes used in modifying DNA were obtained from New England Biolabs, Boehringer Mannheim, or Bethesda Research Laboratories. Oligonucleotides were synthesized by a Systec 1450 or ABI 380A synthesizer. Nucleotide sequencing was done chemically (Maxam and Gilbert, 1979) or enzymatically (Sequenase, U. S. Biochemical Corp.).

A clone of a T7 DNA fragment carrying the complete wild-type coding sequence of genes 4A and 4B was obtained in the anti-sense cloning vector pBR322(A1), but not in pBR322 itself. This clone, pAR3729 (Fig. 1), carries a PstI-PstI fragment (bp 11,483-13,384) of T7 DNA that begins within the gene 2.8 coding sequence 82 bp ahead of the 4A initiation codon and ends within the gene 4.3 coding sequence 121 bp past the shared 4AB termination codon (Fig. 2). This fragment also contains the complete coding sequences of the putative gene 4.1 and 4.2 proteins, whose coding sequences overlap the gene 4 coding sequences in a different reading frame (Fig. 2). The PstI promoter was inserted ahead of the 4AB coding sequence by fusing the appropriate PstI-BamHI fragments from pET-1 and pAR3729 to create pAR3730.

Codon 64 of the 4A coding sequence in pAR3730 was changed from ATG (methionine) to TTA (leucine), thereby eliminating the initiation codon for the 4B coding sequence. At the same time, a silent change was introduced into codon 63 (proline, CCA to CCG) to create a new HpaI site (GGTAA). The total change was from CCA ATG ACT to CCG TTA ACT. This set of changes in 3 base pairs is referred to as the gene 4A-ML64 mutation, and the resulting protein is referred to as the 4A' protein. This mutant 4A' coding sequence was created by removing a 68-bp Bsu36I-HpaI fragment (bp 11,738-11,806 of T7 DNA) from pAR3730 and replacing it with a synthetic double-stranded DNA having the above changes. Replacement involved partial digestion with Bsu36I (two sites in pAR3730, both in gene 4) and complete digestion with HpaI (unique site). Mutant plasmids were detected by colony hybridization, using one of the mutant synthetic DNA strands that had been 5' end labeled with 32P, and identification was confirmed by cutting with HpaI. Determination of the nucleotide sequence of the complete 4A' coding sequence of the mutant plasmid selected for further work (pAR5000) verified that only the above changes had been introduced. The A1 anti-sense promoter was removed by joining appropriate PstI-BamHI fragments from pAR5000 and pBR322 to create pAR5001. The 4A1 or T7lac promoter was inserted ahead of the 4A' coding sequence by fusing the appropriate PstI-BamHI fragments from pET-1 or pET-10 and pAR5001 to create pAR5007 and pAR5018 (see Table II and Fig. 1).

The cloned 4B coding sequence contains bp 11,575-13,384 from T7 DNA, beginning 10 bp downstream of the 4A initiation codon and ending at the same downstream PstI site as the other gene 4 clones. The fragment was made by limited Bal-31 digestion of a BglII-BglII fragment from T7 DNA (bp 11,515-15,253) followed by cleavage with PstI and attachment of BamHI linkers. Cloning this fragment in the BamHI site of pBR322 created pAR3701, in which the nucleotide sequence across the vector/T7 DNA junction at the Bal-31 end was determined. The 4A1 or T7lac promoter was inserted ahead of the 4B coding sequence by fusing the appropriate PstI-BamHI fragments from pET-1 or pET-10 and pAR3701 to create pAR3708 and pAR5019 (see Table II and Fig. 1).

T7 Gene 4 Mutants—Mutations that altered one or both of the gene 4A or 4B coding sequences were constructed in plasmids and then introduced into T7 phage by homologous genetic recombination. In some cases, enrichment for the desired recombinant was accomplished by selecting for a nearby recombination between a mutant phage and wild-type T7 DNA in the plasmid. Restriction analysis of the phage DNA was used where feasible for identifying the recombinant. The correctness of each mutation was confirmed by determining the nucleotide sequence of the mutant phage DNA in the region of the mutation. Pertinent information about the construction and growth of the gene 4 phage mutants is given in Table I.

An amber mutation was placed at the fourth codon of the 4A coding sequence by replacing serine codon TCG with TAG to create the 4A-amS4 mutation. To do this, two polymerase chain reaction products were obtained from T7 DNA by use of Taq DNA polymerase (Cetus) and oligonucleotide primers that introduced the amber mutation (C to A at bp 11,575), an NcoI site CCATGG (G to C mutation at bp 11,563, 2 bp ahead of the 4A initiation codon), and BamHI sites at the ends. The two polymerase chain reaction products, containing bp 11,529-11,568 and 11,552-11,575, were ligated through their NcoI sites and cloned in the BamHI site of pBR322. The mutations were introduced into T7 by recombination with wild type, and 2 out of 650 progeny phage carried the amber mutation, as determined by spot test on BL21 and C1757 (supD).
The 4A-ML64 mutation was introduced into T7 by recombination between the gene 4 amber mutant 4-245 and plasmid pAR5001. About 5% of the progeny phage lost the amber mutation, and HpaI restriction analysis indicated that three out of six of these recombinants acquired the 4A-ML64 mutation.

T7 DNA fragments used for creating deletion mutants were cloned by means of BamHI linkers into the BamHI site of pBR322, but several attempts to obtain a clone of the coding sequence for both the 4A and 4B proteins were unsuccessful, whether directly or by reconstituting the complete gene from separately cloned partial gene fragments. Because we had been successful in cloning almost all T7 genes in this site, some of which are very toxic to the cell (Studier and Rosenberg, 1986), we suspected that something peculiar to the 4A-4B sequence was preventing the cloning.

Based on our previous experience, the most likely impediment to cloning would be the presence of a promoter for E. coli RNA polymerase in the 4A coding sequence ahead of the 4B initiation site. Constitutive expression of 4B protein, known to be toxic, would prevent the clone from being established. Such a promoter would have to be near the beginning of the 4A coding sequence, since the 4B clone we obtained begins only 10 bp downstream of the 4A initiation codon.

The consensus promoter sequence for E. coli RNA polymerase has a −35 sequence of TTGACa and a −10 sequence of TAtaaT, with approximately 16-18 base pairs between the two sequences (Hawley and McClure, 1983 and references therein). Examination of the nucleotide sequence near the 3' end of the possible expression of the putative gene 4.1 protein, whose coding sequence overlaps the 3' end of the 4AB coding sequence in a different reading frame, was unlikely to be the problem because fragments containing these coding sequences could be cloned individually.

We knew from previous work that gene 3.8 is not essential for growth on laboratory hosts and that a gene 3.8 mutant would grow well on BL21. The lysozyme amber mutant 3.5-lys13a (Silberman et al., 1975) was the parental phage for 3.8, and 6 out of 60 heat-treated phage lost the lysozyme mutation and acquired the 3.8 deletion, as determined by ability to grow on BL21 and by restriction analysis of the phage DNAs.

Defective gene 4 deletion mutants require complementing plasmids for growth, but plasmids that supply active gene 4 proteins can be toxic to both E. coli and T7, causing cell death or inhibiting T7 growth to various degrees dependent on both the plasmid and the host. Both HMS174 and C1757 hosts tolerate gene 4 deletion mutants but should not be used to propagate a gene 4 deletion mutant, C1757 strains produce higher titer stocks. Plasmid pAR5018, which supplies 4A' protein, is perhaps the most convenient complementing plasmid for growing gene 4 deletion mutants but should not be used to propagate 3.8-4A, which picks up the 4A' mutation by recombination; pAR3729 is used to propagate 3.8-4A. All of the deletions extend farther into gene 4 than the cloned fragment in the complementing plasmids, so that homologous recombination between complementing plasmid and the deletion phage should not be able to generate wild-type phage. The parental phage for 3.8-4A and 3.8-4B was wild-type T7, and after heat enrichment 13 out of 270 and 81 out of 270 plaques, respectively, were complementing by spot test. The parental phage for 3.5-lys13a was the gene 5 amber mutant 5-28. About 25% of the progeny phage lost the amber mutation, and 14 out of 90 recombinants acquired the deletion, as determined by spot test.

### RESULTS

**Cloning the Natural 4AB Coding Sequence**—We readily obtained a clone of the 4B coding sequence in the BamHI site of pBR322, but several attempts to obtain a clone of the coding sequence for both the 4A and 4B proteins were unsuccessful, whether directly or by reconstituting the complete gene from separately cloned partial gene fragments.

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**TABLE I**

| T7 mutant | Parental phage | Recombining plasmid | Mutation | Propagating host |
|-----------|---------------|---------------------|---------|-----------------|
| 4A- | Wild | pAR5039 | 4A-4S to amber | C1757 |
| am54 | | | | |
| 4A' | 4-245 | pAR5001 | 4A-ML64 | BL21 |
| 3.8 | 3.5-lys13a | pAR5070 | Δ17,163-11,515 | BL21 |
| Δ3.8-4A | Wild | pAR5071 | C1757/pAR3729 | C1757/pAR3729 |
| Δ3.8-4B | Wild | pAR5071 | C1757/pAR5018 | C1757/pAR5018 |
| Δ3.8-4.5 | 5-28 | pAR4893 | Δ17,297-13,623 | C1757/pAR5018 |

The lengths of deletion ranged from 353 to 2,327 bp (Table I), and the lengths of flanking homologies ranged from 500 to 634 bp to the left of the junction and from 234 to 1,780 bp to the right. Lysates were sometimes enriched for the deletion phage by diluting 100-fold in tryptone broth containing 10 mM EDTA and heating at 55 °C for 15 minutes (Studier, 1973a). Lethal deletion mutants require a complementing clone for propagation and can therefore be readily identified by spot tests using complementing and noncomplementing hosts.

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**TABLE II**

| Plasmid | Vector | Upstream T7 promoter | Gene | Relative plating efficiencya on HMS174/Bl21 |
|---------|--------|----------------------|------|----------------------------------------------|
| pBR322 |        | Δ3.8-4.5/Δ3.8-4.5   | WT   | WT   |
| pAR3729 | pBR322(AI) | ΔAB | 4A' | <10−7 | 1 |
| pAR3730 | pBR322(AI) | g10 | 4AB | 0.8 small | 0.8 small |
| pAR5001 | pBR322 | 4A' | 0.6 | 0.5 |
| pAR5007 | PET-1 | g10 | 4A' | 0.1 tiny | 0.1 tiny |
| pAR5018 | PET-10 | TIlac | 4A' | 0.9 | 0.8 |
| pAR5070 | pBR322 | 4B | 0.1 tiny | 0.6 |
| pAR5019 | PET-10 | g10 | 4B | <10−7 | 0.3 small |
| pAR5019 | PET-10 | TIlac | 4B | 0.3 tiny | 0.9 |

* The cloned fragments also carry gene 4.1 and 4.2 and the φ4c and φ4.3 promoters.

* Plaque size was either large, small, or tiny.

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2 F. W. Studier, unpublished results.

3 A. H. Rosenberg and F. W. Studier, unpublished results.
from this clone and leave an active 4A-B coding sequence were unsuccessful.

Cloning the 4A' Coding Sequence—To make a clone that produces the 4A but not the 4B protein, initiation of 4B protein synthesis must be prevented. The 4B initiation codon is located an appropriate distance from a good ribosome-binding sequence in the sequence CAGGAGTAAACCA-ATG. Changing the ATG initiation codon would presumably prevent initiation of 4B protein synthesis but would necessarily change amino acid 64 of the 4A protein, possibly altering its activity. The ribosome-binding sequence could be altered without changing the amino acid sequence of the 4A protein, but such alterations seemed less likely to prevent synthesis of 4B protein completely.

We elected to change the 4B initiation codon from ATG to TTA, which changes amino acid 64 of the 4A protein from methionine to leucine. Leucine was chosen as the replacement amino acid because it is the most likely amino acid to replace methionine in equivalent positions in related proteins, according to the Mutation Data Matrix of Dayhoff et al., 1978. The replacement, referred to as ML64, was made in the cloned 4A-B fragment from pAR3730. Once the 4B initiation codon was removed, the anti-sense promoter could also be removed. We refer to this mutant 4A protein as the 4A' protein. Results in succeeding sections, and the activities of the purified protein (Patel et al., 1992), indicate that the 4A' protein probably has little if any deficiency relative to wild-type 4A protein.

Expression of Gene 4 Proteins from Clones—Clones capable of expressing an active gene 4 protein, whether 4A' or 4B alone or the combination of 4A and 4B, seem to be toxic to the E. coli hosts we have tried, presumably because of basal gene 4 expression. Even when a gene 4 clone can be established in a particular host, maintenance of the plasmid can be a problem. Some combinations of clone and host seem to have an appreciable fraction of dead cells in saturated cultures (Studier, 1991). Of the hosts we have worked with, HMS174 and C1757 seem somewhat less sensitive than BL21 and 011' to gene 4 toxicity.

The gene 4 clones listed in Table 11 all have two promoters that can be used by T7 RNA polymerase, the φ4tc promoter within the gene 4 coding sequence and the φ3 promoter following the coding sequence (Fig. 2). Transcription from these promoters should proceed around the plasmid to produce gene 4 mRNA (Fig. 1), so the cloned genes should be transcribed by the T7 RNA polymerase produced during T7 infection (Studier and Rosenberg, 1981; McAllister et al., 1981). These promoters can also be used to direct expression of the cloned genes in host cells that provide T7 RNA polymerase (Studier et al., 1990). To try to increase expression levels, we also placed the cloned gene 4 fragments immediately downstream of the strong φ10 promoter of pET-1 or the regulated T7lac promoter of pET-10 (Table 11 and Fig. 1). Attempts to produce gene 4 proteins for purification and biochemical characterization have used DE3 lysogens, which provide IPTG-inducible T7 RNA polymerase (Studier and Moffatt, 1986). HMS174(DE3) and C1757(DE3) were used because they are less sensitive to the toxic effects of basal gene 4 expression than BL21(DE3). The compatible plasmid pLysS was used as a source of T7 lysozyme to reduce basal gene 4 expression by T7 RNA polymerase in uninduced cells (Studier, 1991). In general, pLysS was helpful for stabilizing plasmids having gene 4 under control of the strong φ10 promoter but was not needed when gene 4 was under control of the regulated T7lac promoter (Dubendorff and Studier, 1991).

Examples of expression of the 4A' and 4B proteins are given in Fig. 3. As expected, no 4B protein was detectable from the 4A' clone. We have yet to detect any 4B protein from any 4A' construction, whether in a clone or in T7 itself, and we expect that none is produced. The most sensitive tests we have made (Fig. 3B) would have detected at least 1 molecule of 4B protein in 80 molecules of 4A' protein.

The 4A' protein is synthesized at a somewhat higher rate than the 4B protein, and about 10 times as much accumulates after induction (Fig. 3). However, neither the 4A' nor 4B protein is synthesized well compared with many other target proteins that are made efficiently in the T7 expression system, and neither continues to be synthesized at its maximal rate beyond an hour or so. This relatively poor expression is presumably caused by the toxic effects of the accumulating gene 4 protein. Consistent with this interpretation, we have observed that inactive partial or mutant gene 4 proteins can accumulate to considerably higher levels. Nevertheless, expression from the cloned genes produces 40-400 times more gene 4 protein than does T7 infection (compare Kolodner et al., 1978; Fischer and Hinkle, 1980; and Patel et al., 1992).

T7 Mutants Defective in Expression of Gene 4 Proteins—Before having an active gene 4 clone, only conditional-lethal gene 4 mutants could be isolated in T7 itself, because propa-
gation of the phage requires functional gene 4. All of the amber mutants we tested affected both the 4A and 4B proteins, and we did not try to determine whether any temperature-sensitive mutants might be defective in 4A but not 4B.

Tests with gene 4 amber mutants indicated that the 4AB clone pAR3729 can support the growth of gene 4-defective mutants, but experiments with amber mutants are complicated by recombination between the phage mutant and the cloned gene, which produces a significant fraction of wild-type phage. However, this complementing plasmid could be used to isolate and propagate a set of deletion mutants of T7 that entirely or partially eliminate the gene 4 coding sequences (Table I and Fig. 2). Each deletion was made so that homologous genetic recombination between the mutant phage and the complementing clone would be unable to restore an active gene 4 to the mutant. In practice, this means that the deletion mutant must lack homology with at least one end of the complementing clone. Such mutants can be propagated on the complementing host without accumulating a significant fraction of wild-type (or pseudo wild-type) recombinants and can therefore be studied under nonpermissive conditions without any background from wild-type phages or low level suppression of amber mutations.

Construction of the appropriate deletion mutants was facilitated because the genes flanking gene 4, namely genes 3.8, 4.3, and 4.5, are not essential for T7 growth (Studier, 1981). The deletion mutants are named by the extent of the coding sequences they affect, namely Δ3.8, Δ3.8-4A, Δ3.8-4B, and Δ3.8-4.5. The Δ3.8 deletion retains 50 bp ahead of the 4A initiation codon, and this mutant would be expected to make both 4A and 4B proteins. The Δ3.8-4A deletion retains 16 bp ahead of the 4B initiation codon, and this mutant would be expected to make 4B but not 4A protein. The Δ3.8-4B deletion extends 18 codons into the 4B coding sequence, and this mutant should be unable to make either 4A or 4B protein. The Δ3.8-4.5 deletion completely removes all gene 4 coding sequences.

The Δ3.8-4.5 mutant is completely unable to form plaques on normal laboratory hosts but has normal specific infectivity when a host carries pAR3729 (Table II). Presumably, the cloned gene 4 is expressed by T7 RNA polymerase during infection, beginning at the 4c and 4.3 promoters and proceeding around the plasmid (Fig. 1). Transcription from the anti-sense promoter, which is by E. coli RNA polymerase, should have shut off by the time gene 4 proteins would normally be made during infection (Dunn and Studier, 1985). On the other hand, too much gene 4 protein from a clone can be inhibitory to T7 growth, as indicated by the decreased plaque size and/or plating efficiency of wild-type T7 when the clone has a strong φ10 promoter in front of the coding sequences for 4AB, 4A', or 4B (Table II).

The uninduced T7lac promoter, which is blocked by lac repressor (Dubenoff and Studier, 1991), seems to relieve a slight reduction of plating efficiency caused by the cloned 4A' or 4B coding sequences themselves (compare pAR5018 with pAR5001 and pAR5019 with pAR2761 in Table II). This slight reduction in plating efficiency is not accompanied by a decrease in plaque size and seems likely to be a result of an increase in the fraction of dead cells in the culture because of a higher basal level of gene 4 protein. Lac repressor bound at the T7lac promoter would presumably block a transcribing E. coli RNA polymerase (Nakamura and Inouye, 1982; Deuschle et al., 1986a), thereby reducing basal gene 4 expression. However, bound lac repressor would have little effect on a transcribing T7 RNA polymerase that started at the 4c and 4.3 promoters (Giordano et al., 1989), and therefore the uninduced T7lac promoter should have little effect on levels of gene 4 protein produced from the clone during infection.

Clones that supply appropriate amounts of 4A' protein support apparently normal growth of Δ3.8-4.5, but equivalent clones that supply 4B protein support only tiny plaques at low efficiency, a severe growth deficiency (Table II). Clearly, the 4A' protein is functional and can support the growth of T7 in the absence of 4B protein, but 4B protein without 4A protein is insufficient.

Since 4A' protein from a clone can support T7 growth, we expected that a mutant T7 phage that has the 4A-ML64 mutation, and which therefore should make the 4A' protein but no 4B protein, would be able to grow on normal laboratory hosts. We transferred this mutation from the clone into T7 itself by recombination with a gene 4 amber mutant. About half of the progeny that lost the amber mutation gained the ML64 mutation, and the mutant and wild-type type recombinant plaques were indistinguishable. We refer to the mutant as T7-4A', or simply 4A'.

When plated on the complementing host C1757/4A'-pAR5018, purified 4A' mutant phage and each of the deletion mutants had the same specific plating efficiency as wild-type T7. Relative plating efficiencies of these mutant phages on C1757 itself are given in Table III. Δ3.8-4.5, which lacks both the 4A and 4B proteins, cannot make plaques at all. It has no homology with the complementing clone upon which it was propagated, and any recombinant phages that might have picked up an active gene 4 by illegitimate recombination constituted less than 10^{-8} of the population. Similarly, Δ3.8-4B also cannot produce either the 4A or 4B protein and also does not form plaques. However, this mutant has considerable homology with its complementing clone on one end (Fig. 2), so that active gene 4 can be acquired by one homologous and one illegitimate crossover with the complementing plasmid. Such recombinant phages were observed at a frequency of about 10^{-6}. The Δ3.8 and 4A' mutant phages give normal plaques on C1757, and the Δ3.8-4A' mutant gave only tiny plaques at low efficiency. Thus, the results with phage mutants (Table III) and with complementation by gene 4 clones (Table II) agree that phage infection is completely defective when neither 4A nor 4B protein is present, is very defective when 4B but not 4A is present, and is essentially normal when 4A' but not 4B is present.

These results also predict that an amber mutation in the 4A coding sequence upstream of the 4B start would make 4B but not 4A protein in a nonpermissive host and would be unable to grow. We constructed an amber mutation at codon 4 of the 4A coding sequence in a plasmid by changing the TCG serine codon to TAG and inserted it into the phage by recombination. In contrast to Δ3.8-4A', the 4A amber mutant formed plaques on nonsuppressing hosts at normal efficiency, and 

| T7 Primase/Helicase Clones and Mutants |
|--------------------------------------|
| WT | 4A' | 4A-ML64 | 4A | 4B |
|-----|-----|---------|-----|-----|
| C1757 | 1.0 | 1.0 | 1.0 | 1.0 |
| Δ3.8 | Δ11,163-11,515 | + | + | 0.1 tiny | 1.0 |
| Δ3.8-4A | Δ11,163-11,738 | 0 | + | 6.2 x 10^{-7} | 1.0 |
| Δ3.8-4.5 | Δ11,297-14,623 | 0 | + | <10^{-6} | 1.0 |

* Plaque size was either large or tiny. Purified stocks of all these T7 mutants had normal specific infectivity when plated on C1757/4A'-pAR5018.

* End points (bp) of deletions in T7 DNA. Positions of deletions relative to gene 4 are shown in Fig. 2 and discussed under "Results."
with a plaque size that depended on the host: a host such as R594, which has very low amber suppression, gave tiny plaques whereas less stringent hosts such as BL21 or HMS174 gave small to medium plaques. Typically, most of the phage in a plaque remained amber and were not overgrown with wild-type phage. The most likely interpretation is that very low levels of 4A protein are sufficient to allow T7 growth when normal amounts of 4B protein are made.

Expression of Gene 4 Proteins during T7 Infection—Patterns of protein synthesis during infection of BL21 by wild-type T7 and the 4A', Δ3.8-4A, and Δ3.8-4B mutants are shown in Fig. 4. In a wild-type infection, 4A and 4B proteins are made in small amounts about 8–12 min after infection, the same time as other replication proteins such as T7 DNA polymerase (gene 5) and the single-stranded DNA-binding protein (gene 2.5). Patterns of the gene 4 mutants are similar to wild-type except for the gene 4 region of the gel (we are not able to identify the putative 3.8 or 4.1 proteins): the 4A' mutant makes approximately normal amounts of a 4A protein but no apparent 4B protein; Δ3.8-4A makes larger than normal amounts of 4B protein but no apparent 4A protein; and Δ3.8-4B makes no apparent 4A or 4B protein. These are the proteins expected from the genotypes of the infecting phages (Table I11).

An unexpected feature of the protein patterns is the larger than normal production of 4B protein during infection by Δ3.8-4A. Such an increase was not observed in the 4A amber mutant (not shown). The increased rate of 4B synthesis in the deletion mutant seems likely to be the result of translational coupling to the gene 3.5 coding sequence in this mutant: the Δ3.8-4A deletion places the gene 3.5 termination codon 17 base pairs ahead of the 4B initiation codon, just upstream of the 4B ribosome-binding sequence.

To study the effects of 4A' or 4B protein supplied from a clone during T7 infection, C1757 rather than BL21 was used as a host because it is less sensitive to the toxic effects of the gene 4 proteins. The time course of T7 infection in C1757 is delayed a few min relative to that in BL21, as judged by patterns of protein synthesis, rates of replication, and time of lysis (which is not only slightly delayed but also less abrupt). However, the overall progression of T7 infection is similar in the two hosts.

Patterns of protein synthesis during infection of C1757 itself or carrying 4A'-pAR5018 or 4B-pAR5019, where the gene 4 coding sequences are preceded by the T7lac promoter, are shown in Fig. 5. Infection was by wild-type T7 or by the 4A' mutant. The timing and amounts of 4A' and 4B protein produced from these plasmids seem rather similar to the timing and amounts of 4A and 4B protein produced in a normal infection. In the patterns shown in Fig. 5, labeling of the 4A band increased above normal when either phage infected a cell carrying the 4A' clone; the 4B band increased when wild-type infected the 4B clone; and a 4B band appeared when the 4A' mutant infected cells carrying the 4B clone.

Measurements of protein synthesis during infection, combined with plating efficiencies of defective mutants (Tables II and III), suggest that the 4A'-pAR5018 and 4B-pAR5019 clones supply gene 4 proteins during infection at times and in amounts appropriate for growth of phage that lack such proteins and that the increase supplied to a phage which itself makes the protein does not generate a level that becomes inhibitory to growth.

Effects of 4A and 4B Deficiency on Replication—T7 replication is severely deficient when both the 4A and 4B proteins are lacking (Studier, 1969). We tested replication during infection by deletion and point mutants that lack either the 4A or 4B protein.

The 4A' mutant, which lacks the 4B protein, grows essen-

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**FIG. 4.** Time course of protein synthesis by wild-type and gene 4 defective phage. BL21 cells were grown shaking at 30 °C in M9 minimal medium to A600 = 0.5 and infected (multiplicity of infection = 10) with wild-type, 4A', Δ3.8-4A, or Δ3.8-4B. Samples were labeled with [35S]methionine, processed, and analyzed as in Fig. 3A. In each set, samples were labeled for 4 min just before infection and 0–4, 4–8, 8–12, 12–16, and 16–20 min after infection. Positions are shown for the 4A and 4B proteins, and for T7 RNA polymerase (gene 1), T7 DNA polymerase (gene 5), and the T7 single-stranded DNA binding protein (gene 2.5.) (Dunn and Studier, 1983).

**FIG. 5.** Time course of protein synthesis by wild-type and 4A' phage in the presence of 4A' and 4B plasmids. C1757 cells with no plasmid, or carrying 4A'-pAR5018, or 4B-pAR5019 were infected with (A) wild-type or (B) 4A' phage. Growth of cells, infection, time of labeling with [35S]methionine, and processing of samples are the same as in Fig. 4.
tially normally, as indicated by plating behavior and burst size, but it appears to incorporate only perhaps 60% as much \(^{3}H\)thymidine during infection as does wild type (Fig. 6). This mutant also has a slightly delayed lysis, another indicator of possible replication deficiency (Studier, 1969). The apparent rate of replication in BL21 has an early peak and a later peak (Fig. 6), in contrast to a single peak observed in C1757 (Fig. 7) or in B (Studier, 1969). Apparently, the appearance of a single or double peak depends on the host; the reason for the difference has not been determined, but one possibility might be a difference in kinetics of release of unlabeled nucleotides from host DNA. The Δ3.8-4A and 4A amber mutants, which make 4B but not 4A protein, have little detectable replication in BL21 (not shown). Thus, 4A protein is needed for replication, and 4B protein alone is not sufficient. The replication results are entirely consistent with the plating behavior of these mutants, if one assumes that the moderate decrease from wild-type replication levels shown by the 4A' mutant has little effect on burst size.

We wanted to see if supplying 4B protein to the 4A' mutant would restore wild-type levels of replication. We used the 4A-par5018 and 4B-par5019 plasmids in C1757 for these tests, complementing conditions that do not inhibit plating efficiency of wild-type T7 (Table II). As in BL21, incorporation of \(^{3}H\)thymidine during 4A' infection of C1757 is only about 60% that during wild-type infection (Fig. 7). Supplying either 4A' or 4B protein from the clone not only made \(^{3}H\)thymidine incorporation by 4A' and wild-type T7 equivalent, but enhanced incorporation 2–3-fold over the normal wild-type level. Lysis was also accelerated, a further indication of the correlation between the efficiencies of replication and lysis.

A simple interpretation of these relative rates of replication would be that the level of gene 4 protein normally limits the rate of replication and that supplying additional gene 4 protein from a clone relieves this limitation. In this interpretation, the 4A' protein would have no intrinsic deficiency, and the reduced replication in the 4A' mutant would simply reflect a reduced total amount of gene 4 activity because no 4B protein is made.

**DISCUSSION**

Previous work (Bernstein and Richardson, 1989) and results presented in the accompanying paper (Patel et al., 1992), indicate that the gene 4A protein has both primase and helicase activities and that the 4B protein has helicase but not primase activity. Our results demonstrate that 4A protein is required for appreciable replication and growth of T7 and that 4B protein is not required. Thus, the primase activity of the 4A protein appears to be essential, but the helicase activity of the 4B protein is dispensable, presumably because it can be replaced by the helicase activity of 4A.

The rate of replication of wild-type T7 or the 4A' mutant is enhanced by the addition of either 4A' or 4B protein, suggesting that replication during a normal infection is limited by the amount of gene 4 protein. Addition of either 4A' protein or 4B protein would be expected to increase both primase and helicase activity, 4A' because it has both activities and 4B because it has helicase activity and can stimulate the primase activity of 4A (Bernstein and Richardson, 1989; Patel et al., 1992). However, only very small amounts of 4A protein are needed for T7 growth, as demonstrated by the ability of a 4A amber mutant (but not a 4A deletion mutant) to make plaques with normal efficiency on nonsuppressing hosts, in which normal amounts of 4B are made, but 4A protein is made only by basal suppression of the amber mutation. Therefore, primase activity seems unlikely to be limiting normal T7 replication and, by default, helicase activity seems likely to be limiting.

Both the 4A and 4B proteins are very toxic to E. coli cells, and too much of either protein is toxic to T7 growth as well. Since the 4B protein has no primase activity, helicase activity may be primarily responsible for the toxicity of both proteins. Apparently, the levels of the gene 4 proteins must be carefully regulated during T7 infection to have enough to support efficient replication but not so much as to prevent growth. The overlapping 4A and 4B proteins may have evolved to provide an appropriate ratio of helicase to primase activity during infection. If mutants of 4A' that retain primase but lack helicase activity could be isolated, they might permit independent variation of primase and helicase activities and thereby help to define the relative roles of the two activities in replication and toxicity.

The toxicity of the gene 4 proteins made cloning the natural

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**Fig. 6.** Time course of DNA replication by wild-type and 4A' phage. The pattern shows the incorporation of \(^{3}H\)thymidine into acid-precipitable counts during infection of BL21 by wild type (●) or 4A' (O). Cell growth and infection are the same as in Fig. 4. 100 μl of culture was labeled for 2 min with \(^{3}H\)thymidine (84.2 Ci/mmol) before and at 2-min intervals after infection. 2 ml of cold 5% trichloroacetic acid was added to stop the labeling and precipitate the DNA. The precipitate was filtered through Whatman GF/C filters and washed several times with 5% trichloroacetic acid and then 95% ethanol. Filters were dried and counted in a liquid scintillation fluor (RPI corp.).

**Fig. 7.** Time course of DNA replication by wild-type and 4A' phage in the presence of 4A' and 4B plasmids. Patterns show the incorporation of \(^{3}H\)thymidine into acid-precipitable counts during infection by wild type (●) or 4A' (O) of C1757 with no plasmid or C1757 carrying 4A'-par5018 or 4B-par5019 plasmids. Cell growth, infection, labeling with \(^{3}H\)thymidine, and processing of samples are the same as in Fig. 6, except that cells were labeled for 2 min at 4-min intervals after infection.
gene difficult and also appears to limit the amount of protein that can be produced from gene 4 clones. However, sufficient protein can be produced from these clones for biochemical and structural analysis (Patel et al., 1992).

Our solution to obtaining 4A protein without 4B was to change the methionine initiation codon of 4B to leucine. This 4A' mutant protein is produced without detectable amounts of 4B protein and appears to have normal 4A activity in the tests we have made. We chose what appeared to be a conservative analysis of gene-binding region upstream of the protein that can be produced from these clones for biochemical and structural analysis (Patel et al., 1992).

We have not tried to make the wild-type 4A protein by synonymous codon replacements in the ribosome-binding site (Mendelman, L. V., and Richardson, C. C. (1991) J. Biol. Chem. 266, 23240-23250). Our solution to obtaining proteins that have equivalent activities for biochemical and structural analysis (Patel, S. S., Rosenberg, A. H., Studier, F. W., and Johnson, K. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 596-600).

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Note Added in Proof—Mendelman and Richardson have recently analyzed biochemical properties of wild-type 4A protein produced from a clone of gene 4 that has synonymous codon replacements in the 4B ribosome-binding site (Mendelman, L. V., and Richardson, C. C. (1991) J. Biol. Chem. 266, 23240-23250).