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Entry of Bacteriophage T7 DNA into the Cell and Escape from Host Restriction

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T7 DNA did not become susceptible to degradation by the host restriction enzymes *Eco*B, *Eco*K, or *Eco*P1 until 6 to 7 min after infection (at 30°C). During this period, T7 gene *0.3* protein is made and inactivates *Eco*B and *Eco*K, allowing wild-type T7, or even a mutant that has recognition sites flanking gene *0.3*, to escape restriction by these enzymes. However, T7 failed to escape restriction by *Eco*P1 even though *0.3* protein was made, evidently because *0.3* protein is unable to inactivate *Eco*P1. How T7 DNA can be accessible to transcription but not restriction in the first few minutes of infection is not yet understood, but we favor the idea that the entering DNA is initially segregated in a special place. Entry of T7 DNA into the cell is normally coupled to transcription. Tests of degradation of DNAs having their first restriction sites different distances from the end of the DNA indicated that only the first 1,000 or so base pairs (2.5%) of the molecule enter the cell without transcription. An exception was the only mutant tested that lacks base pairs 343 to 393 of T7 DNA; most or all of this DNA entered the cell without being transcribed, apparently because it lacks a sequence that normally arrests entry. This block to DNA entry would normally be relieved by the host RNA polymerase transcribing from an appropriately situated promoter, but the block can also be relieved by T7 RNA polymerase, if supplied by the host cell. T7 mutants that lack all three strong early promoters A1, A2, and A3 could grow by using a secondary promoter.

Bacteriophage T7 contains a linear, double-stranded DNA approximately 40,000 base pairs (bp) long, whose complete nucleotide sequence is known (6, 16). Upon infection, T7 DNA enters the *Escherichia coli* cell left end first (17). Transcription of T7 DNA is entirely from left to right, the first one-fifth of the molecule by the host RNA polymerase and the remainder by newly made T7 RNA polymerase (4, 32). Entry of T7 DNA into the cell appears to be coupled to transcription: when transcription by the host RNA polymerase is prevented by rifampin, T7 DNA does not enter the cell (36); and transcription by T7 RNA polymerase proceeds initially in a wave from left to right down the late region of the DNA, even though promoters for T7 RNA polymerase are distributed throughout the molecule (14). As judged by the pattern of transcription, entry of the DNA molecule is not complete until about 40% of the way through the latent period, and the gradual entry seems to be an important element in control of gene expression (14).

We and others (11, 36) suspected that the gradual entry of T7 DNA into the cell might also be an important element in the ability of T7 to overcome host restriction. T7 grows interchangeably on *E. coli* B, K, and C, because T7 gene *0.3* specifies a protein that binds to and inhibits the *Eco*B and *Eco*K restriction enzymes (1, 12, 27). The gene *0.3* protein is not made until 3 to 4 min after infection (24), but if gene *0.3* (position 2.32 to 3.20 in T7 DNA) were to enter the cell and be expressed before entry of the first recognition site for *Eco*B (position 11.88) or *Eco*K (position 37.96), the restriction enzyme could be inactivated before it would have a chance to act on the entering DNA. The inability of T7 to escape restriction by *Eco*P1 (22) also fits nicely with this model, since nine *Eco*P1 recognition sites lie to the left of or within gene *0.3*.

To test this model and to obtain another measure besides transcription of when different parts of the T7 DNA molecule enter the host cell, we analyzed when the infecting T7 DNA molecule first begins to be degraded by *Eco*P1, *Eco*B, and *Eco*K, using appropriate T7 mutants alone or in the presence of inhibitors of transcription or translation. To our surprise, the results do not support this model for how T7 escapes host restriction but instead suggest that T7 DNA somehow remains inaccessible to all of these restriction enzymes for the first 6 to 7 min of infection, during which time the early region of T7 DNA can be transcribed and translated.

MATERIALS AND METHODS

T7 DNA. General information about T7, its genetic organization, and the functions of genes and genetic signals is given in references 6, 24, and 29, which include references for more detailed descriptions. Positions of genetic elements in the nucleotide sequence of T7 DNA (6, 16) are given as nucleotide number or in units of 1% the length of wild-type T7 DNA (one T7 unit is 399.36 bp), beginning at the genetic left end. Locations of recognition sites for restriction enzymes in T7 DNA were determined by computer search of the nucleotide sequence.

Bacteriophage T7 strains. Wild-type T7, media for growing cultures, and procedures for growing and purifying phage particles have been described previously (23). The deletion mutants used in this work are described (see Fig. 6 and Table 1). Deletion mutants D364 and D353 have been described previously (31); deletion mutants 4101, D502, D503, and Δ28 were isolated in the course of other work (unpublished data). Location in T7 DNA of the D364 and Δ28 deletions was determined from the nucleotide sequence across the point of the deletion; locations of the other deletions were inferred by restriction mapping and by assuming that the deletions arose as a result of crossovers at short nucleotide sequence repeats (31).

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***E. coli* strains.** Bacterial strains not otherwise identified have been described previously (27). W3110 was used as the *Eco*K-restricting host, B or B707 as the *Eco*B-restricting host, and BL21(P1) as the *Eco*P1-restricting host. The P1 strain used to make the BL21(P1) lysogen was P1CM*clr*100 (19). Nonrestricting hosts were C1757, a C strain that carries *supD*; B834, an isogenic derivative of B707 that lacks *Eco*B restriction and modification activity; and BL21 (30), a Met^+ transductant of B834. BL21(DE2) is a lysogen of a lambda derivative in which T7 RNA polymerase is supplied constitutively in amounts sufficient to support the growth of T7 deletion mutants that lack the gene for T7 RNA polymerase (30).

Enzymes and antibiotics. Restriction enzymes, RNase A, RNase T_1, proteinase K, and enzymes and oligonucleotides used in cloning were obtained from New England BioLabs, Inc., Bethesda Research Laboratories, Inc., Boehringer-Mannheim Biochemicals, or Worthington Diagnostics and used according to the instructions of the suppliers. Rifampin and chloramphenicol were obtained from Sigma Chemical Co.

Construction of T7 mutants containing additional *Eco*K sites. *Eco*K sites were inserted into T7 DNA ahead of gene *0.3*, behind it, or on both sides of it. The strategy was to insert a small fragment that contains an *Eco*K recognition site into the appropriate site in a fragment of T7 DNA that had been cloned in a plasmid and then to transfer it into T7 DNA by genetic recombination during growth of T7 on cells containing the plasmid. Details of the constructions have been described by B. A. Moffatt (Ph.D. thesis, University of Toronto, Toronto, Ontario, Canada, 1985).

Briefly, a 20-bp *Hae*II-*Sau*3A fragment from pBR322 DNA (nucleotides 1648 to 1667) (33) was used as a source of the *Eco*K recognition site. After various linkers and adaptors were added, the fragment was inserted into *Alu*I sites at bp 836 or 1379 in T7 DNA, located ahead of or behind the coding sequence of gene *0.3*, bp 925 to 1278. The resulting mutants are designated sRK836 or sRK1379; a mutant containing insertions at both sites is designated sRK836,1379. The insertion at bp 1379 was accompanied by a deletion of bp 1380 to 2128, which eliminates genes 0.4 to 0.7 of T7.

Because of the history of the constructions, the inserted fragments had rather complex linkers that contained alternating *Bam*HI and *Eco*RI sites. The fragment inserted at bp 836 contained restriction sites in the order *Bam*HI-*Eco*RI-*Bam*HI-*Eco*K-*Bam*HI-*Eco*RI-*Bam*HI; the fragment inserted at bp 1379 contained two *Eco*K sites, and the order of sites was *Bam*HI-*Eco*RI-*Eco*K-*Eco*RI-*Eco*K-*Eco*RI-*Bam*HI. Determination of the nucleotide sequence (13) showed that the asymmetric *Eco*K sequence in sRK836 reads GCACCAT TATGTT from 5′ to 3′ in the *l* strand of T7 DNA, and the two *Eco*K sites at bp 1379 are both in the opposite orientation.

Mutants that had picked up the *Eco*K fragment by recombination with the plasmid were identified by their susceptibility to restriction by *Eco*RI or by their resistance to heat inactivation and inability to grow on host strain BR3, properties both conferred by the deletion in sRK1379 (25). The mutants that carried *Eco*RI sites were not sufficiently sensitive to restriction by *Eco*RI to identify by spot test unless the *Eco*RI methylase was defective. Therefore, we used a plasmid that carried a normal *Eco*RI nuclease and a temperature-sensitive methylase, plasmid pDB7, kindly provided by P. Modrich.

A derivative of sRK836 that lacks gene *0.3* function was constructed by crossing sRK836 and the deletion mutant D353. The progeny were heated to inactivate most of the phage that lacked the deletion, and individual plaques were screened for restriction by *Eco*RI (due to the *Eco*RI sites in the inserted fragment) and for restriction by *Eco*K (because the D353 deletion eliminates gene *0.3* function). The double mutant sRK836,D353 was found at a frequency of 0.7%.

Preparation of labeled phage particles. Purified T7 particles containing ^{32}P at relatively high specific activity were needed for assays of degradation of infecting T7 DNA, so that less than one phage per cell could be used and still detect the label in a reasonably short time. Phage were grown at 37°C in C1757, in B2 medium containing 0.16 mM PO_4 (28) supplemented with tryptophan (10 μg/ml). When a growing culture of C1757 reached an optical density at 600 nm (OD_{600}) of 0.10, 100 μCi of $^{32}PO_4$ per ml was added, and the cells were grown until an OD_{600} of 0.50 (just before the PO_4 becomes limiting). Cultures were infected with phage at a multiplicity of 0.1 and shaken until lysis, approximately 1 to 1.5 h. Phage were purified rapidly by precipitation with polyethylene glycol followed by isopycnic banding twice for 35 min at 150,000 × *g* in steps of CsCl solution, first with the phage layered atop the steps and then with the phage layered beneath the steps. The phage band was collected in one-drop fractions into 1.5-ml polypropylene tubes (Eppendorf) containing 10 μl of 1% gelatin, which helps to prevent the labeled phage from sticking to the tube. The titer of the peak fractions was typically between 5×10^{11} and 1.0×10^{12} phage per ml.

T7 phage prepared in this way have an estimated specific activity of 8.3×10^{-11} μCi per molecule, which corresponds to one ^{32}P disintegration per 90 T7 molecules per h. This high rate of disintegration produces a fairly rapid loss of ability to transfer DNA into the host cell, so the phage were used within 6 to 8 h from the time of lysis, when less than 10% of the population should have sustained a disintegration within its DNA. Secondary effects of the radiation seem less important, because the titer of the purified phage did not drop appreciably during this period. Occasionally, labeled phage were prepared which, for unknown reasons, did not inject their DNA efficiently even when assayed soon after purification.

Gel electrophoresis of infecting T7 DNA. This assay is similar to one described by M. W. McDonell (Ph.D. thesis, State University of New York, Stony Brook, 1977). Cells were grown in tryptone broth (23) at 30°C in a shaking water bath to an OD_{600} of 0.45, which generally corresponded to about 3×10^8 cells per ml. A 2-ml culture in a 50-ml flask was infected with ^{32}P-labeled phage at a multiplicity of 0.1 to 0.5 (0.3×10^8 to 1.5×10^8 phage per ml). Typically, more than 85% of the phage was adsorbed. Samples of 25 μl were removed at different times after infection and mixed with 25 μl of 2% sodium dodecyl sulfate–100 mM Tris chloride, pH 6.8–2% mercaptoethanol–4 mM trisodium EDTA–20% glycerol–0.05% bromocresol green–0.05% xylene cyanol (fast) in a 1.5-ml polypropylene tube (Eppendorf). The mixture was immediately placed in a 65°C water bath for 5 min to lyse the cells and release DNA from phage particles. When rifampin was present it was added 5 min before infection to give a concentration of 200 μg/ml; when chloramphenicol was present it was added 1 min before infection to give a concentration of 100 μg/ml. To analyze the labeled DNA, 5 μl of each sample, corresponding to 2.5 μl of original culture, was analyzed by electrophoresis on 0.3% agarose gels in 40 mM Tris-acetate–2 mM trisodium EDTA, pH 8.1, for 1.5 h at 5 V/cm, followed by autoradiography.

Isolation of DNA from T7-infected cells. Cultures grown at

30°C in tryptone broth were infected as described above for gel electrophoresis of infecting DNA. Samples were removed, and the cells were harvested by low-speed centrifugation. The cell pellet was suspended in 0.1 M NaCl–10 mM Tris hydrochloride, pH 8.0–1 mM trisodium EDTA, and the cells were lysed by the addition of 1% sodium dodecyl sulfate and then treated with 10 μg each of RNase T_1 and RNase A per ml for 15 min at room temperature, followed by 50 μg of proteinase K per ml for 1 h at 37°C. After phenol extraction, the DNA was precipitated with ethanol, dried, and dissolved in the buffer appropriate for digestion with the desired restriction enzyme.

Analysis of protein synthesis during T7 infection. Cultures were grown at 30°C in M9 medium to a concentration of about 5×10^8 cells per ml and infected with wild-type or mutant T7 at a multiplicity of about 10. In cases in which infection killed the cell, the fraction of surviving cells measured 4 min after infection was less than 0.1%. Samples of culture were labeled with [^{35}S]methionine (20 μCi/ml) immediately before and at different times after infection, and the labeled proteins were analyzed by electrophoresis through a 10 to 20% polyacrylamide gradient gel in the presence of 0.1% sodium dodecyl sulfate, followed by autoradiography, essentially as described (26).

RESULTS

Fate of infecting T7 DNA. McDonell (Ph.D. thesis) found that the DNA of a gene *0.3* mutant of T7 is degraded after infection of an *Eco*B$^+$ but not an isogenic *Eco*B$^-$ host, using gel electrophoresis to follow the fate of infecting ^{32}P-labeled T7 DNA. We have used this simple assay to determine the time at which infecting DNA that is susceptible to restriction begins to be degraded by the three restriction enzymes *Eco*B, *Eco*K, and *Eco*P1. *Eco*B and *Eco*K are type I restriction enzymes and *Eco*P1 is a type III restriction enzyme (for reviews, see references 2, 7, and 35). All three enzymes initiate action on DNA at specific recognition sequences, but the *Eco*B and *Eco*K endonucleases cut at random sites that are usually some distance away from their recognition sequences, whereas the *Eco*P1 endonuclease cuts at specific sites that are 25 to 27 nucleotides away from its recognition sequence. We expected degradation of the infecting T7 DNA would begin shortly after the first recognition sequence entered the cell, so that the time degradation began would reflect the time of entry of the first recognition site.

Results of a typical experiment are shown (Fig. 1). Host strains having active *Eco*B, *Eco*K, and *Eco*P1 were infected with ^{32}P-labeled wild-type or *0.3*-mutant T7, and samples were analyzed at intervals after infection. In each case, the labeled DNA remained entirely unaffected for the first 5 min after infection, migrating as a single band indistinguishable from the DNA isolated from phage particles. Beginning with the 7.5- or 10-min samples, the intensity of this band decreased, and label began to be found at the origin of electrophoresis. For a productive infection, such as wild-type T7 infecting the *Eco*B or *Eco*K hosts, the movement of label to the origin should represent conversion of parental DNA to replicating forms, which are known to contain long concatemers (9). At late times, most or all of this label returns to the position of mature DNA in the electrophoresis pattern, presumably as a result of packaging into phage particles. On the other hand, when the T7 infection is aborted by restriction, as in wild-type infection of a P1 lysogen or *0.3*-mutant infection of any of the restricting

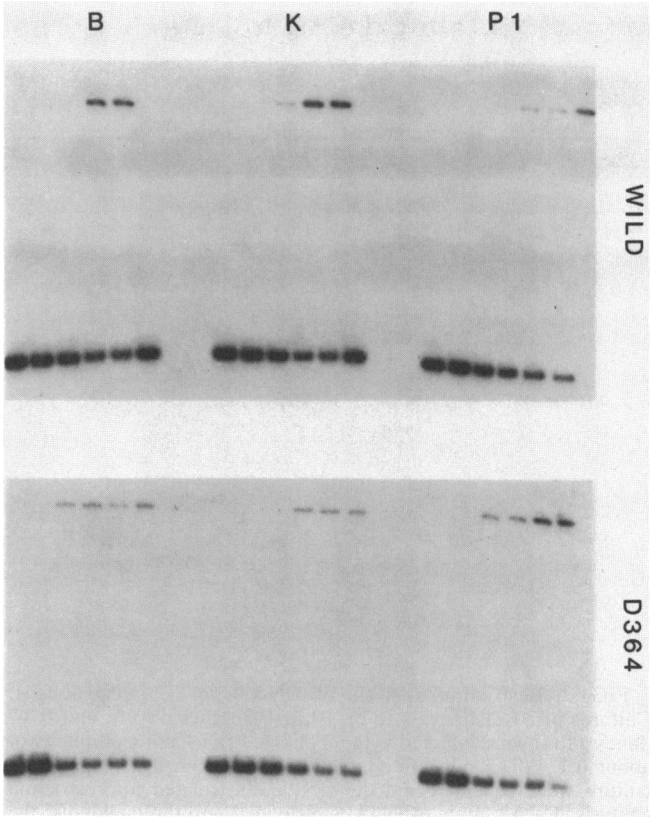

FIG. 1. Gel electrophoresis to monitor the fate of infecting T7 DNA. Cultures of B707 (B), W3110 (K), and BL21(P1) (P1) were infected with ^{32}P-labeled wild-type T7 or the gene *0.3* deletion mutant D364 at a multiplicity of about 0.1. Samples were removed 2.5, 5, 7.5, 10, 15, and 30 min after infection and subjected to electrophoresis through a 0.3% agarose gel followed by autoradiography.

hosts, the label that appears at the origin should represent degradation products of T7 DNA that have been reincorporated into host DNA. When the infection is restricted, the amount of label in mature T7 DNA only decreases, and there is no increase at late times.

The label that remains at the position of mature T7 DNA throughout infection presumably represents DNA in unadsorbed phage particles or in particles that adsorbed but did not inject their DNA. Samples were not fractionated before gel electrophoresis, so all label present in the samples should have been represented in the gel. Typically, more than 85% of the label had adsorbed to the cells. In all experiments of this type, the multiplicity of infection was kept low enough that most cells received only a single molecule of T7 DNA, so the restriction capacity of the cells would not be overloaded. The fraction of DNA molecules that appeared not to be metabolized varied among individual preparations of labeled phage, but increased with the length of time since the phage were labeled, presumably because of increasing accumulation of radiation damage. We generally used phage within 6 to 8 h after they were grown, when less than 10% of the DNA molecules should have sustained a ^{32}P disintegration.

To confirm the identity of the label found at the origin of electrophoresis, we analyzed its susceptibility to *Bam*HI, which cuts host DNA but not T7 DNA, and *Hae*II, which cuts both DNAs. The samples (Fig. 2) were from ^{32}P-labeled

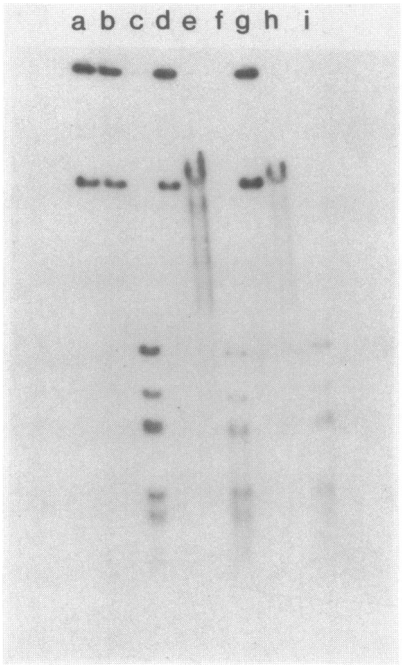

FIG. 2. Restriction analysis of DNA from T7-infected cells. Cultures of BL21 (lanes a to c), BL21(P1) (lanes d to f), and B707 (lanes g to i) were infected with ^{32}P-labeled D364 at a multiplicity of about 0.1. At 15 min after infection, cells from 0.25 ml of each culture were collected and the DNA was isolated for restriction analysis. Each sample of DNA was divided into three aliquots; the first was left uncut (lanes a, d, and g), the second was digested with *Bam*HI (lanes b, e, and h), and the third was digested with *Hae*II (lanes c, f, and i). The digests were subjected to electrophoresis through a 0.3% agarose gel followed by autoradiography.

0.3 mutant T7 particles infecting a nonrestricting host, a P1 lysogen, or an *Eco*B strain. DNA was prepared from samples collected 15 min after infection, and more than half of the label was found at the origin. In the productive infection (lanes a to c), none of the labeled DNA was cut by *Bam*HI but all of it was cut by *Hae*II to produce the pattern of bands characteristic of T7 DNA. However, after infection of the restricting hosts (lanes d to f and g to i), the DNA at the origin was cut by both *Bam*HI and *Hae*II to produce the heterogeneous mixture of fragments characteristic of host DNA, whereas in these same samples the T7-length DNA was not cut by *Bam*HI but was cut by *Hae*II to give the characteristic T7 pattern of bands. Thus, the label found at the origin of electrophoresis in samples from a productive infection is in T7 DNA (presumably replicating forms), whereas label at this position in samples from a restricted infection is in host DNA.

Equivalent experiments (not shown) confirmed that the infecting DNA of wild-type T7 is degraded and incorporated into host DNA after infection of a P1 lysogen, but that it is converted to replicative forms and produces a normal infection in an isogenic *Eco*P1$^-$ strain (kindly provided by W. Arber) or in strains containing active *Eco*B or *Eco*K. Experiments with isogenic host mutants have demonstrated that degradation of the infecting T7 DNA requires active *Eco*B, *Eco*K, or *Eco*P1 (not shown). However, the immediate products of cleavages by these restriction endonucleases were not apparent in gel electrophoresis patterns, presumably because the DNA fragments are rapidly degraded by other nucleases, such as exonuclease V, the *recBC* nuclease (21). Loss of label from the band of infecting DNA is not always accompanied by the appearance of an equivalent amount of label at the origin of electrophoresis (Fig. 1). The most likely explanation for any apparent loss of label is that some of the label is in degradation products that are distributed widely enough to be difficult to distinguish from the background. About 20% of the label from infecting DNA was released as acid-soluble material upon restriction by *Eco*P1 or *Eco*B.

Kinetics of degradation of infecting T7 DNA. The results in Fig. 1 show that when T7 infection is restricted, the infecting T7 DNA begins to be degraded by *Eco*P1 or *Eco*B sometime between 5 and 7.5 min after infection, and it begins to be degraded by *Eco*K slightly later, between 7.5 and 10 min. Other experiments with closer time intervals showed that degradation is initiated by *Eco*P1 or *Eco*B about 6 to 7 min after infection and by *Eco*K about 2 min later. The first recognition sites for *Eco*P1, *Eco*B, and *Eco*K in T7 DNA are 243, 4,746, and 15,160 bp from the left end of the DNA, ahead of any coding sequence, within gene *1*, and within gene *5*, respectively. The later initiation of degradation of infecting DNA by *Eco*K is consistent with a gradual entry of T7 DNA from left to right, but the lack of difference between the time degradation by *Eco*P1 and *Eco*B begins was unexpected.

By 6 to 7 min after infection, the earliest time at which infecting DNA begins to be degraded in the restricting hosts, the entire early region of T7 DNA would have been transcribed and translated in a normal infection (6, 14, 15, 24). This implies that the early region, which contains 24 recognition sites for *Eco*P1 and 1 for *Eco*B, is transcribed and translated well before the time these restriction enzymes would begin to degrade the DNA. To test this directly, we have analyzed patterns of protein synthesis during infections that are restricted by *Eco*P1 or *Eco*B.

The time course of protein synthesis during infections that are restricted by *Eco*P1 or *Eco*B was essentially the same as during a normal infection for the first 6 min, with early protein synthesis beginning normally (Fig. 3). However, synthesis of late proteins was depressed by *Eco*B restriction and completely prevented by *Eco*P1 restriction. The greater severity of *Eco*P1 restriction of protein synthesis is consistent with the difference in restriction of T7 growth; plating efficiency of wild-type T7 on a P1 lysogen was less than 10^{-8}, whereas plating efficiency of gene *0.3* mutants on *E. coli* B was typically 10^{-4} to 10^{-3}. These differences probably reflect the different numbers of recognition sites in T7 DNA, 126 for *Eco*P1 and 6 for *Eco*B. A factor that might contribute to the significant synthesis of late proteins during *Eco*B restriction is the high multiplicity of infection (about 10 in this experiment) needed to assure synchronous infection of every cell. Under such circumstances, it may take longer to degrade every molecule of infecting DNA.

Considering in more detail the effects of *Eco*P1 restriction, the pattern of protein synthesis is entirely consistent with destruction of the template DNA about 6 to 7 min after infection, the time labeled parental DNA was found to be degraded. At this time, the wave of transcription by host RNA polymerase would have reached the end of the early region but little if any transcription by T7 RNA polymerase would have begun. In the *Eco*P1-restricted patterns of Fig. 3, synthesis of the *0.3* and *0.7* proteins reached normal rates, but the rate of gene *1* protein synthesis, while substantial, did not reach the normal rate, and the rate of synthesis of *1.3* protein remained very low. This suggests that destruction of the template DNA occurred when the accumulation of

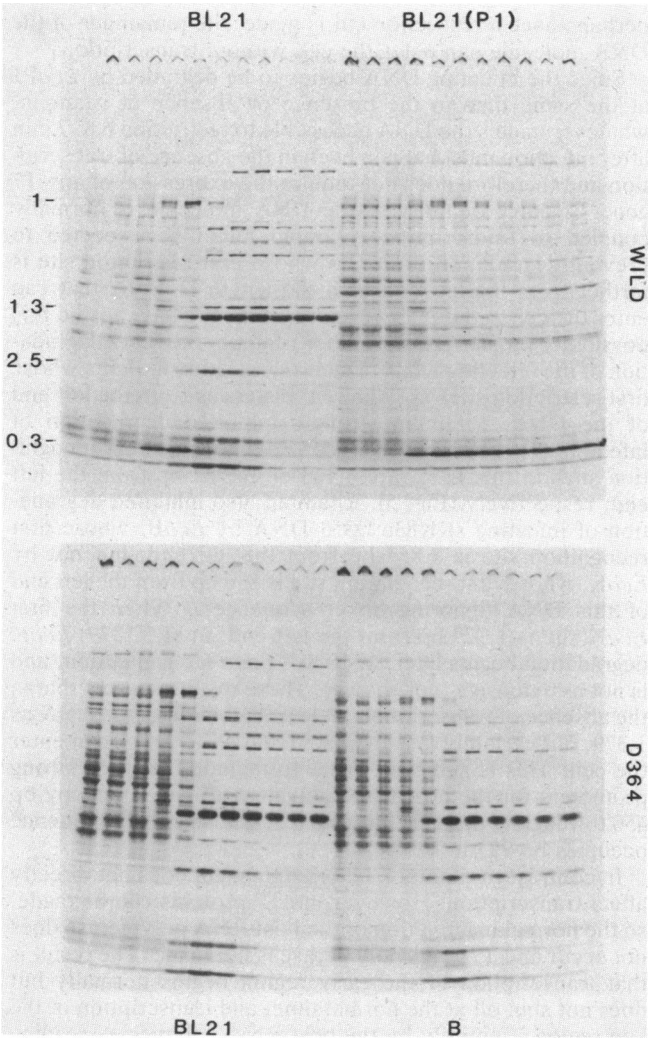

FIG. 3. Protein synthesis during T7 infection of cells having different restriction specificities. Cultures of BL21, BL21(P1), and B were infected with either wild-type T7 or the deletion mutant D364. Samples were labeled with [^{35}S]methionine for 2 min beginning before infection and at 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 min after infection, and the labeled proteins were analyzed by gel electrophoresis. The positions of a few T7 proteins referred to in the text are indicated; other T7 proteins may be identified by comparison with Fig. 3 of reference 6. The three T7 proteins that first become strong bands in the 4- to 6-min pulse of the wild-type infection of BL21 are the gene *1*, *0.7*, and *0.3* proteins; the gene *1.3* protein, which migrates slightly faster than the gene *0.7* protein, does not become labeled significantly until the 6- to 8-min pulse. In these patterns, the gene *9* and *10* late proteins migrate at the same positions as the gene *0.7* and *1.3* early proteins, respectively.

mRNA had reached normal levels for genes *0.3* and *0.7* but not for genes *1* and *1.3*, consistent with an interruption of the rightward wave of transcription across the early region before all of the transcribing RNA polymerases had made it to the end. Synthesis of the gene *0.3*, *1*, and *1.3* proteins, as well as many host proteins, continues well past the usual time of shutoff, even though transcription by host RNA polymerase would be shut off by the action of gene *0.7* protein (3, 20). This synthesis presumably continues because the mRNAs for these proteins are relatively stable and no competing late T7 mRNAs are produced (see also Fig. 8, which shows the decay of protein synthesis after transcription was stopped with rifampin). Little if any synthesis of late proteins can be detected, even though substantial amounts of T7 RNA polymerase, the gene *1* protein, are made in the P1 lysogen. (In some experiments, very small amounts of gene *2.5* protein could be detected.) Apparently, the template DNA was degraded before T7 RNA polymerases could transcribe it to any significant extent.

It is clear that the early region of T7 DNA is transcribed and translated before degradation of the infecting DNA by *Eco*P1 begins, even though there are 24 restriction sites in this region of the DNA. This is also true for restriction by *Eco*B, although in this case only one restriction site lies within the early region, within gene *1*. Apparently, the entering DNA is transcribed for several minutes before it is degraded by these restriction enzymes. During this period of a wild-type infection, the gene *0.3* protein would be made and would inactivate *Eco*B and *Eco*K, thereby allowing T7 to escape restriction. However, T7 does not escape restriction by *Eco*P1 even though *0.3* protein is also made before the DNA is degraded. The most likely explanation is that *0.3* protein is unable to inactivate *Eco*P1.

A T7 mutant containing *Eco*K sites flanking gene *0.3* is not restricted. If T7 escapes restriction by *Eco*B and *Eco*K because gene *0.3* protein is able to be made before any part of the infecting DNA becomes accessible to the restriction enzymes, one would expect that a T7 mutant that carries an *Eco*K recognition site ahead of gene *0.3* would also escape restriction. On the other hand, if overcoming restriction depended on the DNA ahead of gene *0.3* being free of restriction sites, as we had previously expected, a T7 mutant that carries an *Eco*K site ahead of gene *0.3* would not escape restriction.

To test this directly, T7 mutants that contain an *Eco*K recognition site ahead of gene *0.3*, behind it, or on both sides of it were constructed as described in Materials and Methods. All three of these mutant strains make normal plaques with normal efficiency on an *Eco*K-containing host, showing that they are not subject to restriction by *Eco*K. (On the other hand, introduction of one of these *Eco*K sites into a gene *0.3* deletion mutant, thereby increasing the number of *Eco*K sites in the DNA from four to five, decreased the plating efficiency on an *Eco*K-containing host about fourfold, indicating that the *Eco*K site was functional.) Therefore, the ability of T7 to escape restriction by *Eco*K (and presumably by *Eco*B as well) is not due to the absence of recognition sequences ahead of gene *0.3*, but is rather because the DNA somehow does not become accessible to the restriction enzyme until after the early region has been transcribed and gene *0.3* protein has been made.

Restriction of infecting DNA in the presence of inhibitors of transcription or translation. Zavriev and Shemyakin (36) found by shearing adsorbed phage particles from the surface of the cell that the transcription inhibitor rifampin prevents infecting T7 DNA from entering the cell but the translation inhibitor chloramphenicol does not, consistent with the idea that entry of the DNA is coupled to transcription. Our results indicate that, even though transcription is occurring, the entering DNA does not become accessible to restriction until 6 to 7 min after infection. It seems unlikely that transcription itself is preventing accessibility of the restriction enzymes to the DNA, because recognition sites for *Eco*P1 lie ahead of the transcribed region. What then is responsible for the inaccessibility to restriction at early times, and what is it that makes the DNA become accessible? To learn more about this, we looked at the effects of rifampin and chloramphenicol on the degradation of infect-

ing DNAs having restriction sites different distances from the left end.

The first rightward promoter for the host RNA polymerase initiates RNA chains about 500 bp from the left end of T7 DNA, so at least this much DNA must be able to enter the cell without transcription. The first seven *Eco*P1 sites are located between bp 243 and 332, so if any DNA becomes accessible to restriction in the presence of rifampin, *Eco*P1 should be able to cut it. (There are also five *Eco*P1 sites within 500 bp of the right end of T7 DNA, so that any molecules that might begin to enter right end first should also be degraded.) Therefore, we first determined whether labeled wild-type T7 DNA would be degraded upon infection of a P1 lysogen in the presence of rifampin. To be sure to detect any degradation of the DNA, even if only near an end, we prepared DNA at different times after infection, digested it with *Hae*II, and analyzed the fragments by gel electrophoresis. The *Hae*II fragments from the left and right ends of T7 DNA are clearly resolved, so preferential degradation near either end of the DNA would be apparent.

As shown in Fig. 4, infecting T7 DNA was degraded by *Eco*P1 in the presence of rifampin, beginning sometime between 5 and 7.5 min after infection, the same time as in the absence of rifampin (Fig. 1). Furthermore, all parts of the molecule were degraded, and the degradation products appeared to be reincorporated into host DNA, as indicated by a heterogeneous distribution of label at late times. Densitometer traces indicated that in the early stages of degradation the left end of the DNA is lost more rapidly than the right, suggesting that degradation proceeds from left to right. Since the rifampin prevents transcription from bringing the DNA into the cell, it seems possible that the restriction nuclease itself or other nucleases that act on restricted DNA (such as the *recBC* exonuclease) can draw the DNA into the cell. Or perhaps once a restriction cut is made, the remainder of the DNA molecule can enter the cell without transcription.

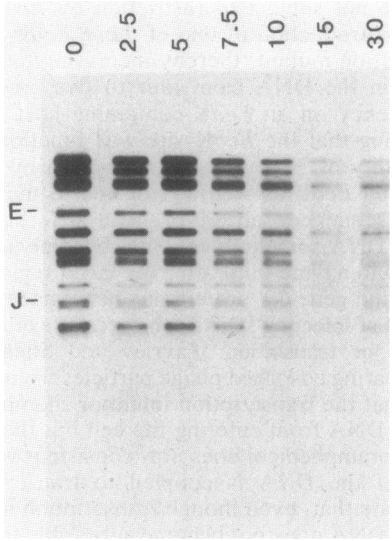

FIG. 4. *Hae*II digestion of labeled DNA isolated after infection of a P1 lysogen in the presence of rifampin. BL21(P1) was infected with ^{32}P-labeled wild-type T7 5 min after the addition of rifampin (200 μg/ml). The multiplicity of infection was about 1. Samples of culture were removed at 0, 2.5, 5, 7.5, 10, 15, and 30 min after infection, and the DNA was isolated, digested with *Hae*II, and analyzed by electrophoresis through a 3% agarose gel followed by autoradiography. The left- and rightmost *Hae*II fragments of T7 DNA (fragments E and J, respectively) (18) are indicated.

Since the infecting DNA begins to be degraded by *Eco*P1 at the same time in the presence or absence of rifampin, whatever makes the DNA accessible to restriction 6 to 7 min after infection must happen even in the absence of transcription and therefore does not require the expression of any T7 gene. If entry of most of the DNA molecule is normally coupled to transcription, rifampin might be expected to prevent degradation of DNAs whose first restriction site is farther from the left end than the length of DNA that can enter the cell without any transcription. We have tested this possibility by analyzing whether rifampin prevents degradation of the infecting DNA of gene *0.3* deletion mutants whose first restriction sites are different distances from the left end of the DNA. Rifampin strongly inhibited degradation of infecting D364 DNA by *Eco*B or *Eco*K, whose first recognition sites in this DNA are 4,168 or 14,582 bp from the left end, respectively (Fig. 5). Rifampin also inhibited degradation of infecting sRK836,D353 DNA by *Eco*B, whose first recognition site is 3,857 bp from the left end, but not by *Eco*K, whose first recognition site is 836 bp from the left end of this DNA (ignoring linker sequences). When the first *Eco*K site is 1,379 bp from the left end, in sRK1379, *Eco*K degradation begins later, at 10 to 12 min after infection, and is not as extensive (not shown). These results suggest that in the absence of transcription, at least 836, perhaps as many as 1,379, but certainly less than 3,857 bp of T7 DNA can enter the cell. This is sufficient DNA to include the three strong promoters for the host RNA polymerase, which occupy bp 450 to 750, and perhaps for gene *0.3*, whose coding sequence occupies bp 925 to 1278.

In contrast to rifampin, chloramphenicol does not directly affect transcription. However, no T7 proteins can be made, so the normal inactivation of the host RNA polymerase does not occur and T7 RNA polymerase is not made. The result is that transcription of the early region begins normally but does not shut off at the normal time, and transcription of the late region is entirely by the host RNA polymerase reading through the termination signal at the end of the early region. Therefore, the early region of T7 DNA should enter the cell normally in the presence of chloramphenicol but the late region should be somewhat delayed, consistent with the relatively slow entry detected by shearing experiments in the presence of chloramphenicol (36).

In the presence of chloramphenicol (not shown), infecting wild-type or *0.3*-mutant DNA was degraded by *Eco*P1 or *Eco*B about 6 to 7 min after infection, about the same time as without antibiotic. The same DNAs were not degraded by *Eco*K until perhaps 15 min after infection, as opposed to 8 to 9 min without chloramphenicol, consistent with a delay in entry of the late region. We expected that when the first *Eco*K site was at bp 836 or 1379 instead of 15160 degradation by *Eco*K in the presence of chloramphenicol would begin about the same time as that by *Eco*P1 or *Eco*B, that is, 6 to 7 min after infection. However, degradation of sRK836 DNA did not begin until about 12 to 15 min after infection, and degradation of sRK1379 DNA did not begin until 10 to 12 min after infection (not shown). This is in contrast to sRK836,D353 DNA in the absence of antibiotics (not shown) or sRK836 DNA in the presence of rifampin (Fig. 5), both of whose DNAs begin to be degraded in the 5 to 7.5 min interval after infection.

The effect of chloramphenicol in delaying degradation when the infecting DNA has its first restriction site at bp 836 or 1379 could be explained if very active transcription can

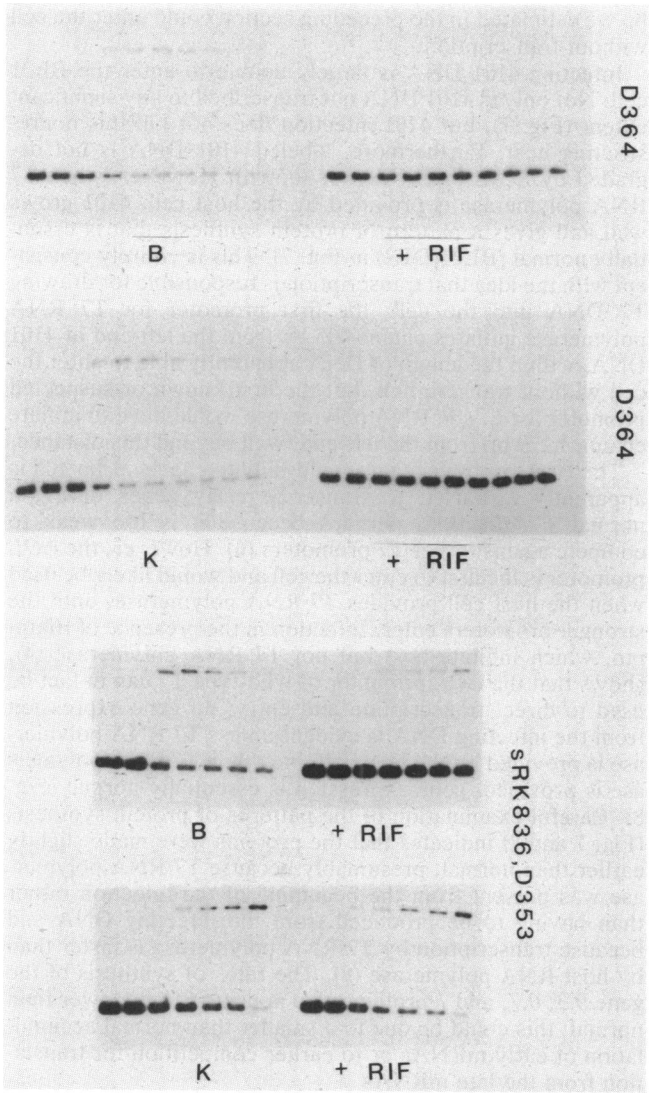

FIG. 5. Effect of rifampin on degradation of infecting DNA by *Eco*B and *Eco*K. Cultures of *E. coli* B (B) or W3110 (K) were infected with ^{32}P-labeled D364 or sRK836,D353 in the presence and absence of 200 μg of rifampin (RIF) per ml, added 5 min before infection. Cultures infected with D364 were sampled at 2, 4, 6, 8, 10, 12, 15, 20, 25, and 30 min after infection. Cultures infected with sRK836,D353 were sampled at 2.5, 5, 7.5, 10, 12.5, 15, and 30 min after infection. Samples were subjected to electrophoresis through a 0.3% agarose gel followed by autoradiography.

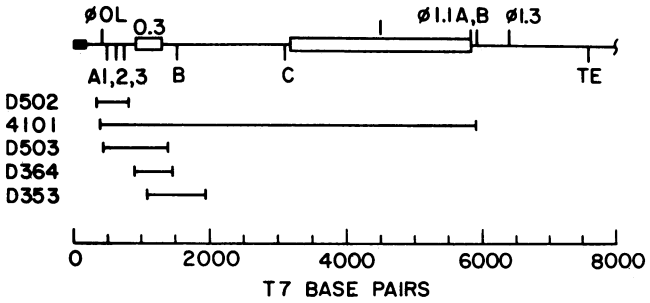

FIG. 6. Locations of deletions relative to promoters for *E. coli* and T7 RNA polymerases within the early region of T7 DNA. Locations of the terminal repetition of T7 DNA (■), the coding sequences of genes *0.3* and *1* (□), and the transcription terminator for *E. coli* RNA polymerase that marks the end of the early region (TE) are indicated. The exact positions of the endpoints of each deletion are given in Table 1.

interfere with restriction. The *Eco*K recognition site at bp 836 lies just downstream of the three strong early promoters, the *Eco*K site at bp 1379 is farther away, and the *Eco*B site at bp 4746 is farther still. Early transcription continues at a high level for at least 10 to 15 min in the presence of chloramphenicol, and perhaps the closer the recognition site is to the promoters the greater the interference with restriction. However, the 6- to 7-min delay in restriction of infecting DNA in the absence of antibiotics is unlikely to be due to interference by transcription, not only because *Eco*P1 sites are found ahead of the transcribed region but also because the same delay is seen in the presence of rifampin, in which transcription is not occurring.

Coupling of transcription and DNA entry. In the course of other work, we have isolated three different deletion mutants of T7 that are useful for providing information about the coupling of transcription and DNA entry during T7 infection. Deletions D502, D503, and 4101 are described in Table 1 and Fig. 6. Each of these deletions removes the A1, A2, and A3 promoters for *E. coli* RNA polymerase that are normally used to initiate transcription of T7 DNA. The D502 deletion extends the farthest to the left, also removing the φ*OL* promoter for T7 RNA polymerase, but does not extend as far right as gene *0.3*. The D503 deletion does not affect φ*OL* on the left but removes gene *0.3* and much of the coding sequence of gene *0.4* on the right. Both D502 and D503 grow normally on nonrestricting hosts, demonstrating that the φ*OL*, A1, A2, and A3 promoters are not necessary for growth. Both strains are restricted by *Eco*B and *Eco*K, showing that gene *0.3*, although present in D502, is not expressed during infection. The 4101 deletion arose by a

TABLE 1. T7 deletions used in this study

Deletion[a]	Base pair position		Length	Repeated sequence	Promoters deleted		T7 genes deleted
	Left	Right			T7	*E. coli*	
D502	342	808	472	AAGACGC	φ*OL*	A1,A2,A3	
4101[b]	393	5,905	5,518	TTAATAC		A1,A2,A3,B,C	*0.3 –1*
D503	423	1,383	964	AAAGA		A1,A2,A3	*0.3 –0.4*
D364	893	1,462	578	CCTTTATGAT			*0.3 –0.4*
D353	1,069	1,952	889	CCGCACT		B	*0.3 –0.7*
Δ28[b]	11,293	11,570	280	CAAT			*3.8*

[a] Each deletion is known or presumed to have arisen by a crossover at the short repeated sequence given (see Materials and Methods); the position given for the left end of each deletion is the last base pair in the left copy of the repeated sequence in wild-type DNA, and the position for the right end is the first base pair in the right copy.

[b] The strain identified as 4101 throughout the paper contains both the 4101 and Δ28 deletions.

crossover between homologies in the ϕOL promoter at bp 405 and the $\phi 1.1$B promoter at bp 5923, leaving an active promoter but removing all of genes *0.3* through *1*. Gene *1* is the only deleted gene absolutely required for T7 growth; however, T7 mutants that lack gene *1* can grow when T7 RNA polymerase is provided by the host cell from the cloned gene (5).

If transcription normally begins at the A1, A2, and A3 promoters, how does transcription and DNA entry proceed in these deletion strains? The patterns of protein synthesis show that the infecting DNA of D502 or D503 is able to be transcribed, but the infecting DNA of 4101 is not (the BL21 patterns shown in Fig. 7). Close examination indicates that the gene *0.7* and *1* proteins are made during the D502 and D503 infections, but, as expected from the inability of these mutants to overcome host restriction, the gene *0.3* protein is not. Since the entire gene *0.3* is present in D502, transcription by the host RNA polymerase must begin after the beginning of gene *0.3* but before gene *0.7* in these strains. The most likely start is at the B promoter for *E. coli* RNA polymerase, a relatively weak promoter that is known to lie in this region. The start site for the B promoter (6) would lie 1,050 bp from the left end of D502 DNA or 547 bp from the left end of D503 DNA, within the length of the 837 to 1,379 bp we estimated in the preceding section could enter the cell without transcription.

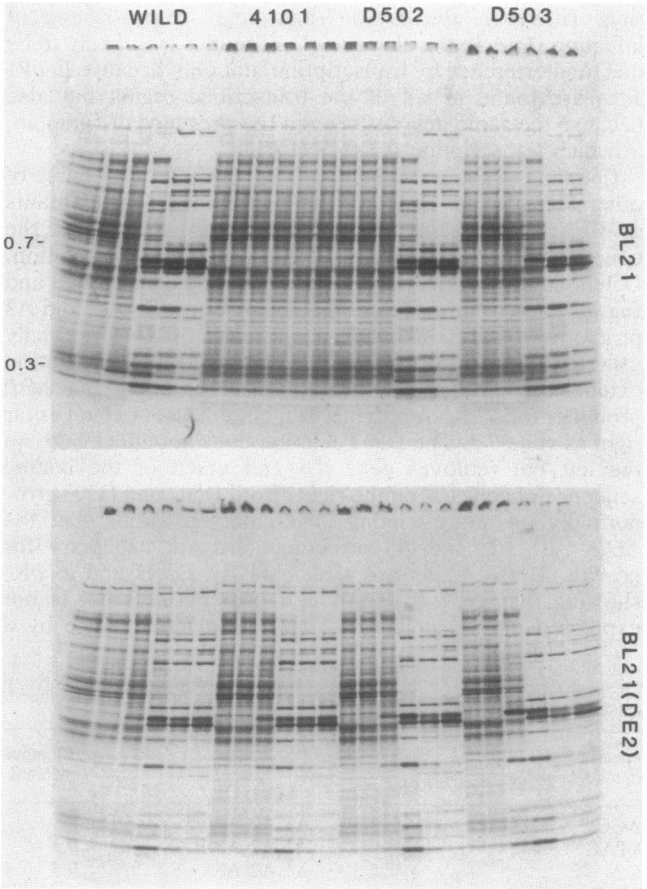

FIG. 7. Protein synthesis during T7 wild-type or deletion mutant infection of a normal host or a host that supplies T7 RNA polymerase. Cultures of BL21 or BL21(DE2) were infected with wild-type T7 or the deletion mutants 4101, D502, or D503. Samples were labeled with [^{35}S]methionine for 4 min beginning before infection and at 0, 4, 8, 12, and 16 min after infection, and the labeled proteins were analyzed by gel electrophoresis.

Infecting 4101 DNA is largely unable to enter the BL21 cell. Not only is 4101 DNA not transcribed to any significant extent (Fig. 7), but 4101 infection does not kill this nonrestricting host. Furthermore, labeled 4101 DNA is not degraded by *Eco*B or *Eco*K (not shown). However, when T7 RNA polymerase is provided by the host cell, 4101 grows well and gives a pattern of protein synthesis that is essentially normal [BL21(DE2) in Fig. 7]. This is entirely consistent with the idea that transcription is responsible for drawing T7 DNA into the cell; the first promoter for T7 RNA polymerase initiates chains 405 bp from the left end of 4101 DNA, within the length of DNA apparently able to enter the cell without transcription, but the first known or suspected promoter for *E. coli* RNA polymerase would have to initiate chains 3,279 bp from the left end, well beyond this distance.

The ϕOL promoter that would initiate chains at bp 405 is apparently not used to transcribe early genes during a normal T7 infection, perhaps because it is too weak to compete against other T7 promoters (6). However, the ϕOL promoter is the first to enter the cell and would likely be used when the host cell provides T7 RNA polymerase, until the stronger promoters enter. Infection in the presence of rifampin, which inhibits host but not T7 RNA polymerase (4), shows that the ϕOL promoter of wild-type T7 can in fact be used to direct transcription and entry: no gene expression from the infecting DNA is evident unless T7 RNA polymerase is provided by the host cell; but when T7 RNA polymerase is provided, gene expression is essentially normal (Fig. 8). Careful examination of the patterns of protein synthesis (Fig. 7 and 8) indicates that the proteins were made slightly earlier than normal, presumably because T7 RNA polymerase was present from the beginning of the infection rather than having to be produced from the infecting DNA and because transcription by T7 RNA polymerase is faster than by host RNA polymerase (8). The rates of synthesis of the gene *0.3*, *0.7*, and *1* proteins also appeared to be lower than normal; this could be due to a smaller than normal accumulation of early mRNAs or to earlier competition for translation from the late mRNAs.

The ability of T7 DNA to enter the cell in the presence of rifampin (when T7 RNA polymerase is supplied) indicates that there is no intrinsic requirement for transcription by *E. coli* RNA polymerase. All of the transcription needed for DNA entry or gene expression can be provided by T7 RNA polymerase.

A mutant in which DNA entry and transcription are uncoupled. 4101, D502, and D503 can all express their genes essentially normally in the presence of rifampin if T7 RNA polymerase is provided by the cell (Fig. 8). This is easily explained for 4101 and D503, because both have a promoter for T7 RNA polymerase 405 bp from the left end of the DNA. However, transcription and entry of D502 DNA was not expected because the first promoter for T7 RNA polymerase in this DNA is the $\phi 1.1$A promoter, which is 5,527 bp from the left end of the DNA, far beyond the 836 to 1,379 bp that should be able to enter the cell without transcription.

Contrary to our results with all other T7 strains analyzed, a substantial amount, perhaps the entire D502 molecule, appeared capable of entering the cell without being transcribed. When T7 RNA polymerase was supplied in the presence of rifampin, the gene *0.3*, *0.7*, and *1* proteins were not made (Fig. 8), indicating that the 5,527 bp ahead of the $\phi 1.1$A promoter enter the cell without being transcribed. Furthermore, the infecting D502 DNA molecule was de-

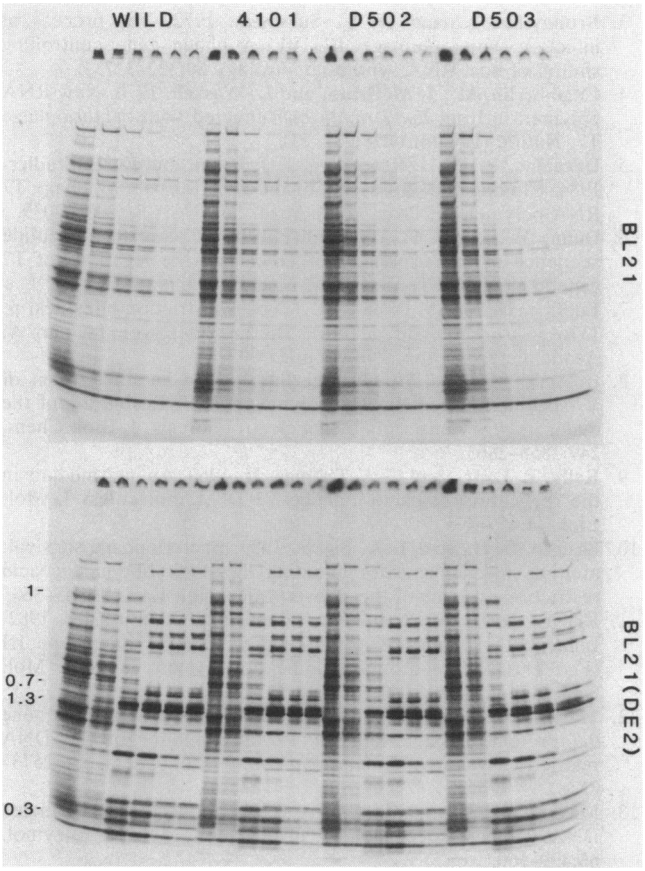

FIG. 8. Protein synthesis during T7 wild-type or deletion mutant infection of a normal host or a host that supplies T7 RNA polymerase, in the presence of rifampin. Cultures of BL21 and BL21(DE2) were grown and infected as described in the legend to Fig. 7, except that 200 μg of rifampin per ml was added to each culture 5 min before infection.

graded by *Eco*B or *Eco*K in the presence of rifampin whether or not the host supplied T7 RNA polymerase (not shown). Thus, at least 37% of the D502 DNA molecule, to the first *Eco*K site 14,521 bp from the left end, appears able to enter the cell without being transcribed. However, this uncoupling of entry and transcription does not make the infecting DNA become accessible to restriction earlier than the 6 to 7 min after infection seen with other T7 strains.

What makes the entry of D502 DNA into the cell different from that of other T7 DNAs? D502 is the only strain that lacks the ϕOL promoter, but this promoter could not be involved in coupling DNA entry and transcription in a normal infection because the DNA must be transcribed before T7 RNA polymerase can be made. However, the D502 deletion also removes bp 343 to 393, upstream of the ϕOL promoter, which are not removed by any of the other deletions. This is an A+T-rich sequence that extends almost to the repeated sequences that are adjacent to the terminal repetition of T7 DNA (6). Perhaps this sequence is needed to arrest the entry of the DNA, providing a block that is normally relieved by the initiation of transcription.

DISCUSSION

The mode of entry of T7 DNA into the cell is clearly an important part of the mechanism by which T7 escapes restriction by *Eco*B and *Eco*K, but not in the way we and others had previously supposed (11, 36). The lack of recognition sites in the nucleotide sequence ahead of gene *0.3* is apparently not required, since we found that T7 escapes restriction by *Eco*K even when recognition sites are placed flanking the gene. Rather, T7 DNA appears to be entirely inaccessible to these restriction enzymes for the first 6 to 7 min of infection, during which time gene *0.3* is expressed and inactivates them (1, 12, 27).

T7 DNA is also inaccessible to *Eco*P1 in the first 6 to 7 min of infection but is then rapidly degraded even though normal amounts of gene *0.3* protein have been made. Apparently, *Eco*P1 is not inhibited by gene *0.3* protein. The opposite conclusion was reached by Krüger et al. (11) primarily on the basis of measurements of acid solubility. However, we found that considerable label from infecting DNA can be reincorporated into host DNA, and in fact we observed about the same amount of acid-soluble label released from infecting wild-type DNA as they reported (20%). Furthermore, they were unable to demonstrate that gene *0.3* protein could inhibit the nuclease activity of *Eco*P1 in vitro (unpublished data of D. H. Krüger, C. Levy, and T. A. Bickle, cited in reference 10). It seems clear that *Eco*P1 cannot be inhibited by gene *0.3* protein in vivo or in vitro and that it does degrade the infecting DNA, as expected for a restriction endonuclease.

How T7 DNA can be accessible to transcription but not to restriction in the first 6 to 7 min of infection remains a mystery. Several mechanisms are conceivable.

(i) Transcription of the infecting DNA could be protecting the DNA from the restriction enzymes. This seems unlikely because several recognition sites of *Eco*P1 lie in the untranscribed region ahead of the early promoters and degradation by *Eco*K begins about the same time in the presence or absence of rifampin, when the *Eco*K recognition site lies within an actively transcribed region in the first 1,000 bp of the DNA.

(ii) *Eco*B, *Eco*K, and *Eco*P1 are relatively complex enzymes whose activity requires or is stimulated by *S*-adenosylmethionine and ATP (2, 7, 35). Perhaps their mechanisms of action are such that 6 to 7 min is required before the first cuts in the DNA can be made, which provides enough time for gene *0.3* protein to be made and for the inactivation of *Eco*B or *Eco*K (but not *Eco*P1). This possibility could be eliminated if such a lag in degradation did not occur with other phage DNAs, but we are not aware that this information is available for other phages.

(iii) Perhaps a protein of the T7 phage particle could enter the cell, somehow protect the DNA for a limited period, and still allow transcription. It is not clear how such protection would work; we have little evidence for or against such a mechanism.

(iv) Although there is no direct evidence supporting it, we currently favor a model in which the incoming T7 DNA initially enters a compartment of the cell that is accessible to RNA polymerases but not to restriction enzymes. A highly speculative version would have the DNA initially remaining outside of the inner membrane of the cell. The RNA polymerases would have to become associated with the membrane in order to transcribe the DNA, sending the transcripts out the cytoplasmic side, and the restriction enzymes would be in the cytoplasm unable to penetrate the membrane. About 6 to 7 min after infection, the portion of the DNA molecule that had been drawn out of the phage particle by transcription (or any DNA that had emerged in the absence of transcription) would be internalized in a process that

would not require the DNA to have been transcribed or any T7 gene products to have been made. This model could also provide a plausible explanation for the block to entry that seems to be located at bp 343 to 393 of the T7 DNA; this sequence might associate specifically with an inner membrane protein of the cell and anchor the DNA until transcription begins. The model would also explain how the linear T7 DNA escapes degradation by exonuclease V, the *recBC* nuclease, which is inactivated by a T7 late function (34).

Whatever the means for protecting T7 DNA in the early stages of entry, our results are consistent with previous observations (14, 36), indicating that entry of the T7 DNA molecule is normally coupled to transcription. The single exception is D502, the only mutant lacking bp 343 to 393, most or all of whose DNA can enter without being transcribed. Apparently, a specific sequence that has been deleted in this strain normally arrests entry of the DNA until the block is relieved by initiation of transcription. Our results indicate that about the first 1,000 bp of T7 DNA (2.5% of the molecule) can enter the cell in the absence of transcription, as judged by the ability eventually to become susceptible to restriction. If a suitable promoter is located within this region, transcription begins and the DNA enters normally. However, if the first promoter is too far beyond this region, transcription cannot begin and the remainder of the DNA cannot enter.

The D502, D503, and 4101 deletion mutants are the first that we know of that remove all three of the strong early promoters for *E. coli* RNA polymerase (A1, A2, and A3) or the ϕOL promoter for T7 RNA polymerase. The ability of these mutants to grow demonstrates that none of these promoters nor any sequence as far left as bp 343 is essential for T7 growth under at least some of the conditions we have available. Secondary promoters for *E. coli* RNA polymerase allow the growth of D502 and D503, but 4101 lacks the gene for T7 RNA polymerase and requires that this enzyme be provided by the host cell in order to grow. When T7 RNA polymerase is provided by the host cell, the ϕOL promoter can be used to initiate transcription and entry of the DNA, completely bypassing the need for transcription by *E. coli* RNA polymerase. The ability to use either polymerase to initiate transcription and entry of the DNA should make it possible to construct viable T7 strains that can be used to define the sequences necessary to arrest DNA entry and to determine how close a promoter for either *E. coli* or T7 RNA polymerase must be for transcription to be able to relieve the block.

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