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Regulation of Expression of Cloned Bacteriophage T4 Late Gene 23

KENNETH A. JACOBS AND E. PETER GEIDUSCHEK*

Department of Biology, University of California at San Diego, La Jolla, California 92093

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The parameters governing the activity of the cloned T4 gene 23, which codes for the major T4 head protein, were analyzed. Suppressor-negative bacteria carrying wild-type T4 gene 23 cloned into plasmid pCR1 or pBR322 were infected with T4 gene 23 amber phage also carrying mutations in the following genes: (i) *denA* and *denB* (to prevent breakdown of plasmid DNA after infection) and (ii) *denA*, *denB*, and, in addition, 56 (to generate newly replicated DNA containing dCMP) and *alc/unf* (because mutations in this last gene *a*llow *l*ate genes to be expressed in *c*ytosine-containing T4 DNA). Bacteria infected with these phage were labeled with ^{14}C-amino acids at various times after infection, and the labeled proteins were separated by one-dimensional gel electrophoresis so that the synthesis of plasmid-coded gp23 could be compared with the synthesis of other, chromosome-coded T4 late proteins. We analyzed the effects of additional mutations that inactivate DNA replication proteins (genes 32 and 43), an RNA polymerase-binding protein (gene 55), type II topoisomerase (gene 52), and an exonuclease function involved in recombination (gene 46) on the synthesis of plasmid-coded gp23 in relation to chromosome-coded T4 late proteins. In the *denA*:*denB*:56:*alc/unf* genetic background, the phage chromosome-borne late genes followed the same regulatory rules (with respect to DNA replication and gp55 action) as in the *denA*:*denB* genetic background. The plasmid-carried gene 23 was also under gp55 control, but was less sensitive than the chromosomal late genes to perturbations of DNA replication. Synthesis of plasmid-coded gp23 was greatly inhibited when both the type II T4 topoisomerase and the host's DNA gyrase are inactivated. Synthesis of gp23 was also substantially affected by a mutation in gene 46, but less strongly than in the *denA*:*denB* genetic background. These observations are interpreted as follows. The plasmid-borne T4 gene 23 is primarily expressed from a late promoter. Expression of gene 23 from this late promoter responds to an activation event which involves some structural alteration of DNA. In these respects, the requirements for expressing the plasmid-borne gene 23 and chromosomal late genes are very similar (although in the *denA*:*denB*:56:*alc/unf* genetic background, there are significant quantitative differences). For the plasmid-borne gene 23, activation involves the T4 gp46, a protein which is required for DNA recombination. However, for the reasons presented in the accompanying paper (Jacobs et al., J. Virol. **39**:31-45, 1981), we conclude that the activation of gene 23 does not require a complete breakage-reunion event which transposes that gene to a later promoter on the phage chromosome. Ways in which gp46 may actually be involved in late promoter activation on the plasmid are discussed.

In the preceding paper (10), we have shown that T4 late genes carried on plasmid or phage vehicles can complement T4 mutants in the same genes. Complementation between cloned viral genes and other virulent phages has been observed (e.g., T7 phages; 5). It has special significance for phage T4 late genes, principally because their transcription is thought to have such elaborate requirements, which apparently include activation of promoters by replication or other DNA "processing." Complementation of the cloned T4 gene 23 apparently does not require the plasmid-borne gene to be recombined to a late promoter carried on complete phage chromosomes (10). That encourages one to believe that the plasmid-borne gene 23 might be acting as an independent unit of transcription. To the extent that this unit is structurally dif-

ferent from a normal phage chromosome, the rules governing its expression should be examined. At least, it might be possible to falsify current hypotheses about T4 late promoter activity; at best, new insights might develop from the analyses of these structurally special T4 late genes.

In this paper, we examine the parameters governing the synthesis of the major T4 head protein, gp23, coded by its cloned gene. The effect of the T4 *alc/unf* gene on this T4 late gene expression has been examined (mutants in the *alc/unf* gene *a*llow T4 *l*ate genes to be expressed in *c*ytosine-containing DNA; 25). Effects of DNA replication and replication proteins on expression of cloned gene 23 have been analyzed, using DNA polymerase (gp43), single-stranded DNA binding protein (gp23), and DNA topoisomerase (gp52) mutants as well as metabolic inhibitors of replication. The effect of a mutation in gene 46 has also been examined (gp46, which determines an exonuclease activity, is absolutely required for forming intermediate structures of T4 recombination; 3). The synthesis of gp23 is shown to require the function of the late-gene-regulatory RNA polymerase-binding protein, gp55.

MATERIALS AND METHODS

Bacterial strains, phage mutants, plasmids, and media. *Escherichia coli* strains K802, CR63, and B834S are described in the preceding paper (10). *E. coli* CSR603 (*thr-1 leuB6 proA2 phr-1 recA1 argE3 thi-1 uvrA6 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33* λ^- *supE44*), obtained from B. Bachmann, was used for the "maxicell" experiments (22). T4 mutant alleles are listed in Table 1 or in the preceding paper (10). Multiple T4 mutants were constructed and their genotypes were verified as described in the preceding paper (10). The *denA* mutation was again screened by measuring host DNA degradation but the following, more sensitive, assay was developed. *E. coli* CR63 were grown at 37°C for three generations in M9S medium (1) supplemented with 0.1 mM uracil and 7.5 μCi of [5-^{3}H]uracil per ml to label both RNA and DNA (the latter as [5-^{3}H]cytosine). The labeled cells were then infected with the T4 mutant of interest. Degradation of the bacterial chromosome was measured by the loss of acid-precipitable counts after alkali treatment to degrade RNA. Phage which were *denA*$^+$ degraded the bacterial DNA releasing [5-^{3}H]dCMP. This label cannot be incorporated into replicating T4 DNA because it is displaced either upon hydroxymethylation of dCMP or upon methylation of derived dUMP (28). The multiple mutant *nd*28:*r*PT8:*am*E51:*unf*39 was constructed from *nd*28:*r*PT8:*unf*39, which had been generously provided by D. P. Snustad.

Plasmids are listed and described in the preceding paper (10), with the following exceptions: pJVH065 contains a *Hin*dIII-generated segment of T4 DNA, covering genes 7 through 12, cloned into pBR313 (30) and was kindly provided by J. Velten; pTE55 contains the T4 *Eco*RI insert of pVH517 (covering T4 gene 24) recloned into the *Eco*RI site of pBR322 (27) and was kindly provided by T. Elliott.

Phage stocks were grown in *E. coli* CR63 at 37°C in M9 (1) supplemented with tryptophan (20 μg/ml),

TABLE 1. *T4 replication genes and mutants*

Gene	Allele	Gene product, function, or mutant phenotype[a]
12	N69[b]	Subunit of short tail fiber
23	H11[b]	Major capsid protein
32	HL618[b]	Single-stranded DNA binding protein; DNA replication, repair, recombination (DO)
43	E4301[b]	DNA polymerase (DO)
46	B14[b,c]	Exonuclease function; concatemer formation in DNA replication and recombination (DA)
52	H17[b,d]	Subunit of T4 topoisomerase (DD)
56	E51[b]	dCTPase-dUTPase
55	BL292[b]	RNA polymerase-binding protein; transcriptional control
denA	*nd*28	Endonuclease II; initiates host DNA degradation
denB	*r*IINB3034, *r*IIPT8[e]	Endonuclease IV; degrades cytosine-containing T4 DNA
unf	*unf*39[f]	(Delayed *unf*olding of host nucleoid; late transcription in cytosine-containing T4 DNA allowed)
denA:*denB*:56:*unf*	*nd*28:*r*PT8:E51:*unf*39	

[a] Mutant phenotypes are listed in parentheses. DA, DNA synthesis arrested after some time; DD, DNA synthesis delayed; DO, no DNA synthesis.

[b] Amber mutation.

[c] From D. Pribnow.

[d] From B. Guttman.

[e] These nearly coextensive deletions also cover adjacent genes including *ndd* (see reference 10).

[f] From D. P. Snustad.

thymidine (12.5 μg/ml), cytosine (12.5 μg/ml), guanosine (12.5 μg/ml), and 2'-deoxyadenosine (25 μg/ml). For experiments in which infected cells were labeled with a ^{14}C-amino acid mixture, phage were pelleted and suspended in 10 mM Tris-hydrochloride, pH 7.5, containing 10 mM $MgCl_2$, 50 mM NaCl, and 0.2% (wt/vol) gelatin. Residual debris was removed by treating the suspended phage with Celite.

Labeling of plasmid-coded proteins in maxicells. *E. coli* CSR603 were transformed with plasmid DNA extracted from *E. coli* K802. These plasmid-containing derivatives of CSR603 were grown in M9S to 2 × 10^8 cells/ml, irradiated with UV light, and shaken overnight. In the morning, the cultures were pelleted and suspended in sulfur-free medium containing 10 μCi of L-[^{35}S]methionine. After a 1-h incubation, samples were prepared for gel electrophoresis and autoradiography as described below. This method, which was devised and is more completely described by Sancar and co-workers (22), specifically labels proteins coded by plasmid-borne genes. Proteins labeled in maxicells were assigned to T4 genes on the basis of the following criteria. The known genetic constitution of the plasmid as determined by marker rescue tests and, in certain instances, complementation tests, allowed us to consider which T4 proteins might conceivably be synthesized, leaving aside such crucial considerations as promoter accessibility. Comigration with T4 proteins was taken as an indication of correspondence; molecular weight standards were included so that comparison could be made with the listed molecular weights of T4 proteins (29, 32). The bands of these reference proteins—bovine plasma albumin (66,000), egg albumin (45,000), chymotrypsinogen (25,000), β-lactoglobulin (18,400), and egg lysozyme (14,300)—were stained with Coomassie blue and remained visible even after treatment of the gel with fluor.

Phage infection and analysis of viral proteins. All experiments were done with *E. coli* B834S containing the plasmid of interest. The *strA* mutation in this strain reduces the ribosomal suppression of amber mutations. Bacteria were grown overnight at 37°C in M9 medium supplemented with 50 μg of methionine per ml and either 20 μg of kanamycin or 10 μg of ampicillin per ml. In the morning, the cells were diluted in the same medium (with or without antibiotic) and grown at 37°C to 2 × 10^8 cells/ml. After the addition of tryptophan (to 40 μg/ml), the culture was shaken at 30°C for 5 min and infected with T4. The multiplicity of infection was 8 to 10 except as noted in the figure legends. To label the proteins, 1-ml portions were withdrawn from the culture (at 30°C) and incubated with 1 μCi of ^{14}C-labeled amino acid mixture. After an appropriate interval, each sample was transferred to 0.2 ml of ice-cold 20% Casamino Acids. Labeled cells were then pelleted, suspended in 50 μl of water, mixed with 50 μl of 0.125 M Tris-chloride, pH 6.8, containing 10% β-mercaptoethanol, 6% sodium dodecyl sulfate, 20% glycerol, and 0.02% bromophenol blue, and lysed by heating in boiling water. To quantitate recovery of infected cells (in Fig. 3–10), 5-μl samples were taken before pelleting and after suspension and were spotted onto glass fiber filters that had been soaked in 10% trichloroacetic acid. The filters were washed with 2.5% trichloroacetic acid–70% ethanol, dried, and counted in toluene-based scintillation fluid. In the experiments shown here, variations in this recovery were compensated when applying samples to gels. Thus, in a single experiment, the amount of protein applied to each lane of a gel came from the same number of cells, labeled in the same volume, with the same amount of ^{14}C-labeled amino acid mixture, compensated only for variations of sample recovery during preparation.

Labeled proteins were resolved by electrophoresis at room temperature on sodium dodecyl sulfate-polyacrylamide gels according to Laemmli (12) and O'Farrell and Gold (20). The concentrations of acrylamide in the gels were 10% (Fig. 2, 3, 7–9), 12.5% (Fig. 1a), or 10 to 12.5% (linear gradient) (Fig. 1b, 4–6, and 10). After electrophoresis, gels were fixed in 45% (vol/vol) methanol and 10% (vol/vol) acetic acid and prepared for fluorography as described by Laskey and coworkers (2, 13) or by using a proprietary fluor (EnHance; New England Nuclear Corp., Boston, Mass.). The gels were then dried on Whatman no. 3 filter paper and exposed to Kodak X-Omat XR5 film at −80°C. The two fluorographic procedures gave equivalent results.

In each figure, T4 proteins are indicated by the numbers of the corresponding genes. They have been identified principally by comparison with the autoradiograms of Vanderslice and Yegian (29).

Materials. ^{14}C-protein hydrolysate mixture (average, 300 μCi/μmol) was purchased from Schwarz/Mann (Orangeburg, N.Y.); [5-^{3}H]uracil (27 mCi/μmol) was from New England Nuclear Corp.; 2'-deoxyadenosine, thymidine, cytosine, guanosine, uracil, 5-fluorodeoxyuridine (FUdR), and hydroxyurea were from Calbiochem (La Jolla, Calif.); ampicillin, tetracycline, kanamycin, and the protein molecular weight markers were from Sigma Chemical Co. (St. Louis, Mo.). L-[^{35}S]methionine was generously provided by M. Miller. All other chemicals were standard reagent grade.

RESULTS

Expression of cloned, late T4 genes in uninfected *E. coli* maxicells and in E. coli after T4 infection. In the first experiment, we examined labeled *E. coli* maxicells carrying the various T4 plasmids for the presence of any T4 proteins. The T4 genes contained within each clone have been identified by marker rescue tests and by some complementation tests (10, 15, 30, 35; Fig. 1; K. A. Jacobs and E. P. Geiduschek, unpublished data. Marker rescue tests, of course, do not prove the integrity of terminal genes in a cloned restriction fragment). The proteins labeled in the uninfected maxicells were assigned to T4 genes on the basis of comigration with well-resolved T4 proteins and molecular weight markers subject to the supposition that they might conceivably be synthesized if the conjugate gene were physically located on the plasmid.

Our marker rescue tests confirmed that the

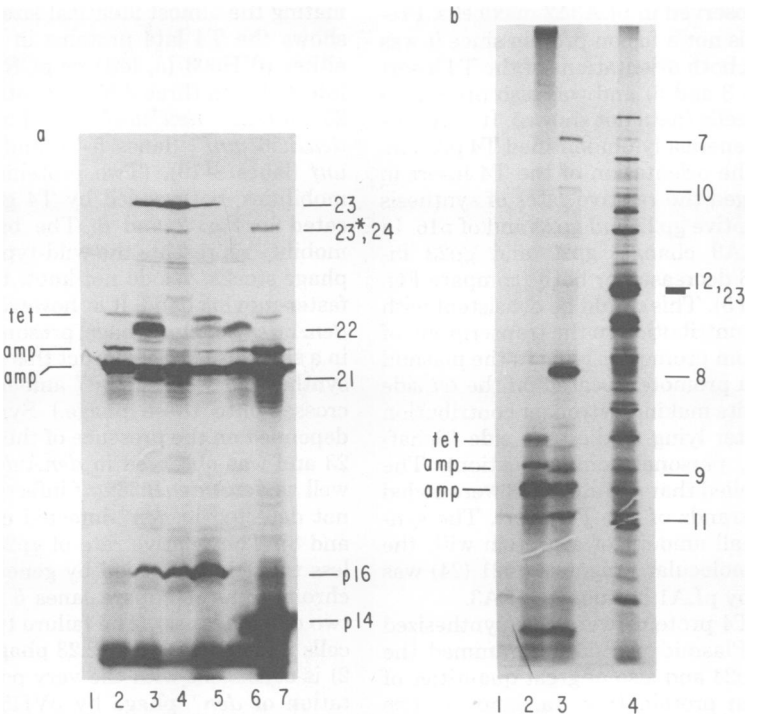

FIG. 1. *Cloned late genes of bacteriophage T4 can be expressed in UV-irradiated E. coli. After UV irradiation, cultures of E. coli CSR603 and its plasmid-carrying derivatives were labeled with L-[^{35}S]-methionine. The labeled proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and detected by fluorography. (a) Lane 1, no plasmid; lane 2, pBR322; lane 3, pLA1; lane 4, pLA4; lane 5, pLA3; lane 6, pLA3Δ2; lane 7, pTE55. (T4 markers not shown.) (b) Lane 1, no plasmid; lane 2, pBR313; lane 3, pJVH065; lane 4, T4 late proteins labeled with ^{14}C-amino acids 25 to 30 min after infection (for comparison of positions of bands only; relative intensities reflect the nature of the label as well as relative rates of synthesis). T4 proteins are indexed on the right. Asterisks indicate post-translational cleavage products.*

T4 insert in pVH503 includes genes 21, 22, and 23 (35). Nevertheless, only T4 gp22 could be assigned unequivocally in a pVH503-maxicell labeling. This is due to an unfortunate property of pCR1-carrying maxicells which synthesize many different proteins (data not shown). We do not know whether each is a primary translation product (8), but this vehicle background can make assignment of insert-specific proteins difficult. We therefore used the pLA series of T4 plasmids in pBR322, which shows a simpler background in maxicell experiments (Fig. 1). pBR322 offers the advantage of a completely known sequence (27). However, A. Schaffermann, R. Kolter, and D. R. Helinski have shown that genes inserted into the *Eco*RI site of pBR322 can be expressed from external promoters in either orientation (personal communication).

The synthesis of a protein with the molecular weight of gp22 was programmed by pLA1 but not by pLA4 from which the *Hin*dIII-*Eco*RI fragment covering gene 21 and a part of gene 22 had been removed (Fig. 1a, lanes 3 and 4). Thus, gene 22 must be entirely located in pLA1 (and consequently in pVH503, from which pLA1 is derived). The synthesis of a protein with the molecular weight of gp23 was programmed by pLA4 and pLA3 but not by pLA3Δ2 (Fig. 1a, lanes 4–6). A minute amount of protein with the molecular weight of gp23*, the processed gp23, was also seen with pLA4 and pLA3. Marker rescue tests showed that pLA3Δ2 still contained the amino terminus of gene 23, but we could not detect the 23 deletion peptide in maxicells, probably because it is unstable in maxicells (although it can be spotted in unirradiated phage-infected cells; see reference 10, Fig. 4). The presumptive gp22 was made in pLA3Δ2 maxicells, as expected (lane 6).

The T4 *Eco*RI fragment transferred from pVH503 into the pLA1 and -3 plasmids also codes for a 16,000-dalton protein (p16 in Fig. 1a). The corresponding coding sequence must be located in or near gene 23 because p16 synthesis was also programmed by pLA5 (data not shown)

but was not observed in pLA3Δ2 maxicells. Presumably, p16 is not a fusion protein since it was synthesized in both orientations of the T4 insert (Fig. 1a, lanes 3 and 5) and was also present in pVH503 maxicells (data not shown). It may correspond to a genetically unidentified T4 protein.

Reversing the orientation of the T4 insert in pBR322 changed the relative rates of synthesis of the presumptive gp22 and gp23 and of p16. In the pLA1–pLA3 change, gp22 and gp23 increased or p16 decreased or both (compare Fig. 1a, lanes 3 and 5). This would be consistent with a substantial contribution to the transcription of these genes from promoters lying in the plasmid vehicle, with a promoter located on the *tet* side of the *Eco*RI site making a stronger contribution than a promoter lying to the *amp* side (Schafferman et al., personal communication). The result also implied that p16 and gp23 were coded off opposite strands of the T4 insert. The synthesis of a small amount of a protein with the approximate molecular weight of gp21 (24) was programmed by pLA1 but not by pLA3.

Other late T4 proteins were also synthesized in maxicells. Plasmid pTE55 programmed the synthesis of gp24 and also of great quantities of a 14,000-dalton protein (Fig. 1a, lane 7). The 14,000-dalton protein probably was a fusion product: maxicells programmed with pVH517 did not make it, but produced a 20,000-dalton protein instead (data not shown). Plasmid pJVH065, which rescued markers in T4 late genes 7 through 12, also programmed the synthesis of gp7 through gp12 (Fig. 1b, lane 3).

To summarize the outcome of these maxicell experiments, they showed the synthesis of several T4 late proteins and of two proteins which are candidates for products of previously undetected genes. The late proteins, gp22 and gp23, were made in cells with plasmids that contained the T4 insert in both orientations. The experiments leave unsettled the question of just how much of these proteins can be made in unirradiated and uninfected cells in which there is more lively competition between genes and mRNA molecules for the transcriptional and translational capacities of the cell. For example, when D. Shibata (personal communication) UV irradiated a λ-*ind*$^-$ lysogen of *E. coli* and then infected it with λ596-29, a recombinant but homoimmune phage (31) carrying T4 genes 50-12, he did not see the conjugate proteins being synthesized.

Synthesis of gp23 could also be followed when unirradiated bacteria containing cloned wild-type gene 23 were infected by T4 gene 12:23 mutants. (The gene 12 mutation is inserted here to simplify reading of the gels for gp23 by eliminating the almost identical-size gp12). Figure 2 shows the T4 late proteins in cells containing either pVH503 (a, left) or pCR1 (b, right) and infected with three different categories of T4 12:23 mutants: *den*$^+$:*unf*$^+$:56$^+$ (lanes 2–4), *denA*:*denB*:56$^+$:*unf*$^+$ (lanes 5–7), and *denA*:*denB*:56:*unf* (lanes 8–10). (Two proteins with different mobilities, both coded by T4 gene 7, are indicated in Fig. 2 and 6. The band with lower mobility represents the wild-type protein of our phage stocks. We do not know the origin of the faster-moving band. It is, however, unique to the *denA*:*denB*:56:*unf* phage, presumably originated in a stock used to construct this strain and is not synthesized when gene 7 amber mutations are crossed into these phage.) Synthesis of gp23 depended on the presence of the cloned T4 gene 23 and was observed in *denA*:*denB*-infected as well as *denA*:*denB*:56:*unf*-infected cells but was not detected in *den*$^+$-infected cells (lanes 2, 5, and 8). The relative rate of gp23 synthesis was less than that afforded by gene 23 on the viral chromosome (compare lanes 5 and 8 with the two outside lanes). The failure to detect gp23 in cells infected with T4:12:23 phage (Fig. 2a, lane 2) is consistent with the very poor complementation of *den*$^+$ phage by pVH503 (Table 6 in reference 10). For reasons that we do not understand, plasmids pLA1 and pLA4 provided better complementation of T4:23 phage. (As far as we know, no analysis of plasmid DNA degradation after T4 infection has yet been made. pCR1 and pBR322 might not be equally stable after infection.) When cells carrying pLA1 or pLA4 were infected by T4:12:23 phage, a small amount of gp23 could be detected (data not shown).

When the infecting phage carried a mutation in gene 55 (lanes 4, 7, and 10), gp23 synthesis could not be detected. This is evidence that the late form of T4-modified RNA polymerase makes the plasmid-derived gene 23 mRNA at this time after infection (30 to 35 min). Synthesis of gp23 also depended on the T4 DNA polymerase (gp43) in *denA*:*denB* but not entirely in *denA*:*denB*:56:*unf* infection (Fig. 2a, lanes 6 and 9). In the latter case, only the plasmid-borne gene 23 escaped the gp43 control: the other late proteins were not synthesized (or, more properly speaking, were made at so low a rate at this time after infection and at this multiplicity of infection that they were not detected here). A more complete analysis of the regulation of the cloned gene 23 in *denA*:*denB* and *denA*:*denB*:56:*unf* genetic backgrounds is presented below. We have focused in particular on the role of various T4 replication proteins.

Regulation of cloned gene 23 after infection with *denA*:*denB*:56$^+$:*unf*$^+$ phage. In

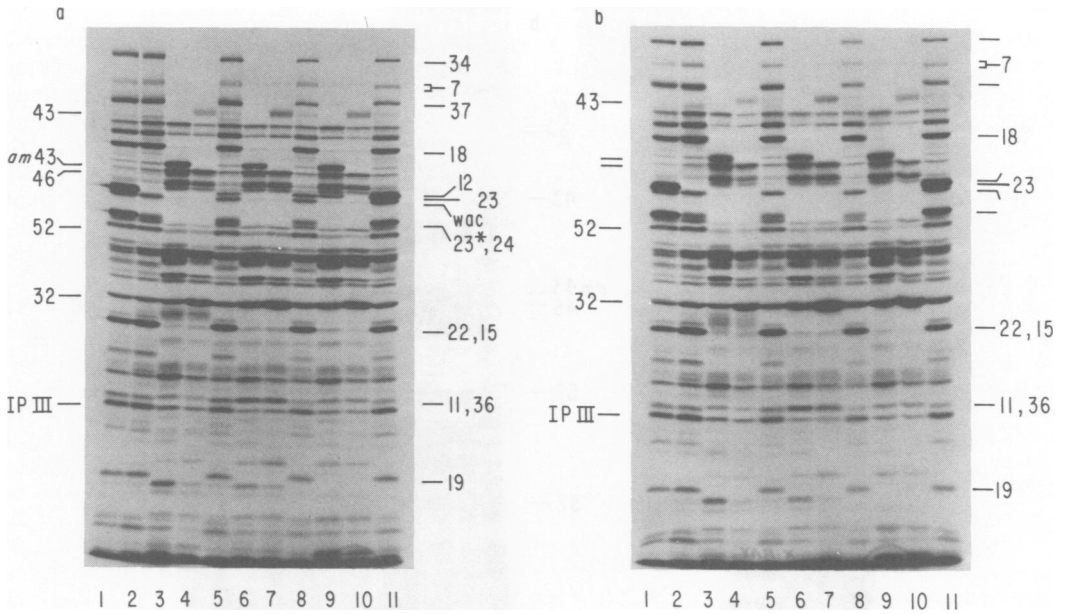

FIG. 2. *Expression and regulation of cloned gene 23 after T4 infection. In this and all subsequent experiments, E. coli carrying various plasmids were infected at 30°C with different T4 mutants and labeled with a ^{14}C-amino acid mixture. The labeled proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (migration from top to bottom in each figure) and detected by fluorography as described in Materials and Methods. In this experiment, the cells carried plasmid pVH503 (a) or pCR1 (b) and were labeled 30 to 35 min after infection with various T4 mutants. Lane 1, denA:denB; lane 2, 12:23; lane 3, 12:23:43; lane 4, 12:23:55; lane 5, denA:denB:12:23; lane 6, denA:denB:12:23:43; lane 7, denA:denB:12:23:55; lane 8, denA:denB:56:unf:12:23; lane 9, denA:denB:56:unf:12:23:43; lane 10, denA:denB:56:unf:12:23:55; lane 11, denA:denB:56:unf. In this and all subsequent figures, early proteins are indexed on the left and late proteins are indexed on the right. Asterisks indicate post-translational cleavage products.*

previous experiments, the complementation-mediated production of phage by *denA*:*denB*:23 phage-infected cells was seen to lag slightly behind production of the complementation-independent *denA*:*denB* phage-infected control cells (Fig. 2a in reference 10). For the corresponding analysis of protein synthesis, cells were pulse-labeled at different times after infection. Figure 3a shows the other late proteins and gp23 first appearing at about the same time, but the third and fourth columns from the left suggest a relatively slight lag for appearance of gp23. The time course of gp23 synthesis after infection with *denA*:*denB*:43 (Fig. 3b) and *denA*:*denB*:55 (Fig. 3c) phage is also shown. When a mutation was introduced either into gene 43 or into gene 55, other late proteins were synthesized in greatly reduced proportions and after a long lag relative to the control (Fig. 3a), as previously observed in *den*$^+$ genetic backgrounds (33). The absence of gp23 signifies that the plasmid-borne gene 23 required gp43 and gp55 for its transcription at least as stringently as did the chromosome-borne late T4 genes. We address this question again below.

The next experiment examined the effects of mutations in other replication genes on synthesis of gp23 programmed by the cloned gene 23. Cells containing pVH503 were infected with *denA*:*denB*:replication-defective mutants at a low multiplicity (see Fig. 4, legend) to reduce any possibility of replication-uncoupled late gene expression (cf. Fig. 3). Cells were labeled from 19 to 24 and from 35 to 40 min after infection. With the interesting exception of the gene 46 mutation (lanes 5 and 11), synthesis of gp23 programmed by the cloned gene responded as did the phage-carried late genes. Synthesis of plasmid-borne gp23 was again seen to depend on gp43 (lanes 3 and 9) and also on gp32 (lanes 4 and 10). Synthesis of gp23 was also delayed and reduced in a gene 52 mutant, together with the other late proteins (compare lanes 6 and 12 with lanes 2 and 8). Identical relationships were seen with plasmid pLA1 or pLA4 in place of pVH503 (data not shown).

The effect of the gene 46 mutation on gp23 synthesis was selective and quantitatively striking: little if any gp23 was made, although the other late proteins were made at high relative

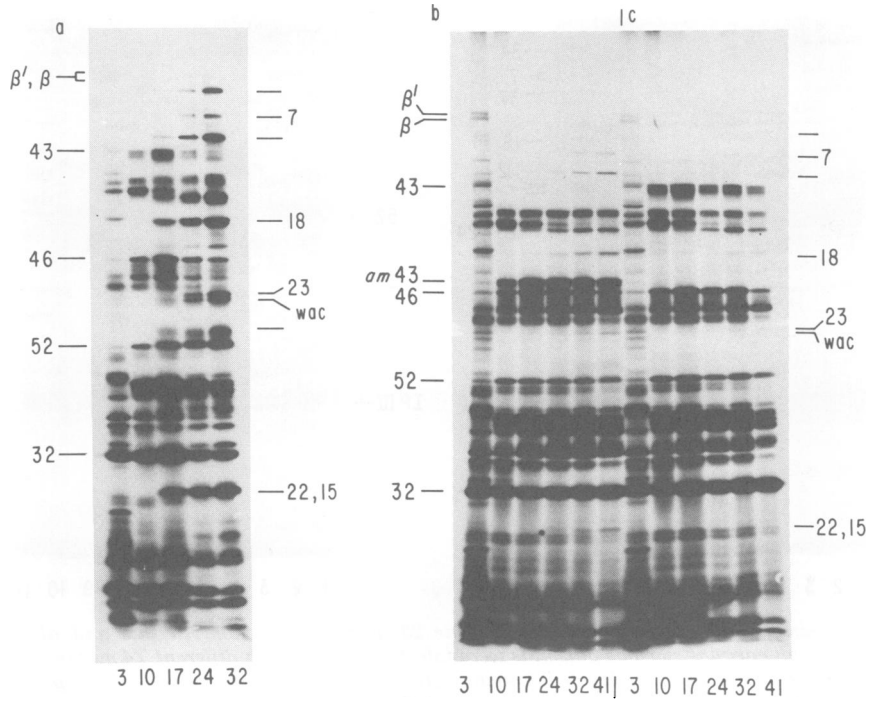

FIG. 3. *Time course of T4 late protein synthesis: contributions from the viral chromosome and from pVH503. E. coli B834S(pVH503) was labeled for 5-min intervals at various times after infection. The times noted in the figure are the beginnings of these labeling intervals. The T4 mutants were: (a) denA:denB:12:23; (b) denA:denB:12:23:43; (c) denA:denB:12:23:55.*

rates. (There appeared to be enhanced labeling in a band migrating just ahead of gp23, but this was not gp23 or gp23 related, since it could also be seen in cells carrying no plasmid, pBR322, or pLA3Δ2 instead of pVH503.)

The preceding experiment suggests that either DNA replication or the replication proteins themselves are equally necessary for expression of cloned late genes and for T4 chromosomal late genes in the *denA:denB*:56$^+$:*unf*$^+$ genetic background. To help distinguish these alternatives, infected cells were treated with FUdR to inhibit thymidylate synthetase or with hydroxyurea to inhibit ribonucleotide reductase. In *denA:denB* phage-infected cells, these treatments block DNA synthesis because the replication complex is starved for one or more deoxynucleoside triphosphates. The block was at least 96% complete for addition of FUdR or hydroxyurea at early times (as judged by incorporation of exogenous [^{3}H]thymidine into DNA; data not shown). Both agents severely inhibited, but did not abolish, synthesis of plasmid- and chromosome-carried late proteins if added 5 min after infection (Fig. 5, lanes 2 and 4). When the addition of hydroxyurea was delayed until 25 min after injection, incorporation of thymidine into DNA continued at a fractional rate of 24% (data not shown); neither FUdR nor hydroxyurea substantially inhibited late protein synthesis if added at 25 min (Fig. 5, lanes 3 and 6). The FUdR block could also be reversed by addition of thymidine (Fig. 5, compare lanes 4 and 5). These results are generally consistent with experiments showing significant levels of late RNA synthesis in FUdR-treated cells (21).

In summary, in a *denA:denB* background, the plasmid-borne gene 23 behaved like the superinfecting late genes in these respects: kinetics of expression and dependence on the activity of several phage DNA replication proteins and on DNA replication. Expression of the cloned gene 23 differed from that of phage chromosomal late genes in requiring gp46. gp46 is part of, or controls, an exonuclease activity that is involved in T4 DNA concatemerization and recombination (reviewed in reference 3).

Regulation of cloned gene 23 after infection with *denA:denB*:56:*unf* phage. Amber mutations in genes 43, 42, 46, and 52 had the same effect on expression of chromosomal late genes in *denA:denB* and in *denA:denB*:56:*unf* genetic backgrounds. However, there were qualitatively and quantitatively different effects on

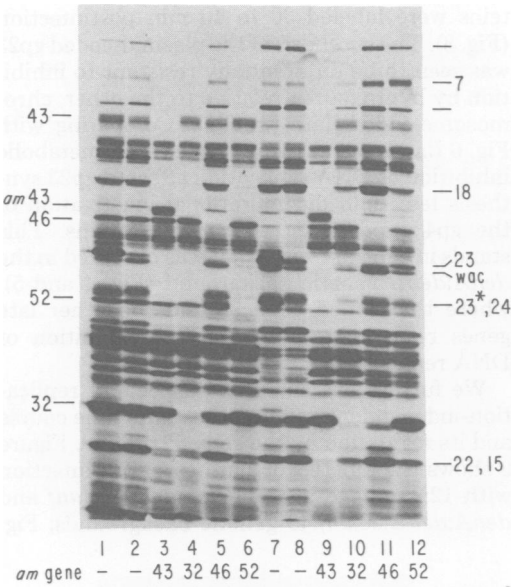

FIG. 4. *Expression of chromosomal genes and of the cloned gene 23 after infection by denA:denB:replication-defective phage. E. coli B834S(pVH503) was infected five times at successive 1-min intervals with one phage/bacterium and then superinfected at a multiplicity of three. Cultures were labeled 19 to 24 min (lanes 1-6) or 35 to 40 min (lanes 7-12) after the first addition of phage. The infecting phage were: (lanes 1 and 7) denA:denB and (lanes 2-6 and 8-12) denA:denB:12:23:X. X includes: (lanes 2 and 8) none; (lanes 3 and 9) 43; (lanes 4 and 10) 32; (lanes 5 and 11) 46; (lanes 6 and 12) 52.*

expression of the cloned T4 late gene 23 (Fig. 6, lanes 2–6 and 8–12, compared with Fig. 4). Nonsense mutations in genes 43, 32, and 46 reduced, but did not eliminate, gp23 synthesis relative to the DNA replication-positive control (Fig. 6, compare lanes 3–5 with lane 2 and lanes 9–11 with lane 8). The residual synthesis of gp23 was proportionately much higher than that of the late proteins programmed by chromosomal T4 genes and could already be detected 19 to 24 min postinfection. The properties of the gene 52 mutant were quite striking. Unlike all of the other late proteins, synthesis of gp23 was not delayed (Fig. 6, compare lanes 2 and 6). Similar results were obtained with plasmids pLA1 and pLA4 (data not shown).

When the T4 topoisomerase is inactivated by mutations in gene 39, 52, or 60 (8, 14), T4 DNA replication is completely dependent on the host DNA gyrase (16). When pLA1- or pLA4-carrying cells were infected with *denA:denB*:56:*unf*:12:23:52 phage in the presence of coumermycin (an inhibitor of *E. coli* DNA gyrase), synthesis of all of the other late proteins was entirely abolished, but some gp23 continued to be made (Fig. 7). The drastic effect of coumermycin on the other late proteins was seen only in the gene 52 mutant genetic background (Fig. 7, compare lanes 5 and 6). In this genetic background, coumermycin did appear to reduce gp23 synthesis (compare lanes 6 and 8) and certainly reduced the rate of gp23 synthesis relative to the rate of gp32 synthesis; it is interesting that the synthesis of one protein (double-headed arrow in Fig. 7) was greatly enhanced (compare lane 6 with lanes 5 and 8). This appeared to be an early protein, and its enhanced synthesis, relative to other early proteins, could already be detected 9 to 14 min postinfection (compare lane 2 with lanes 1 and 4). It had approximately the mobility expected of gp11, but we judge it not to be gp11 because of the timing of its synthesis.

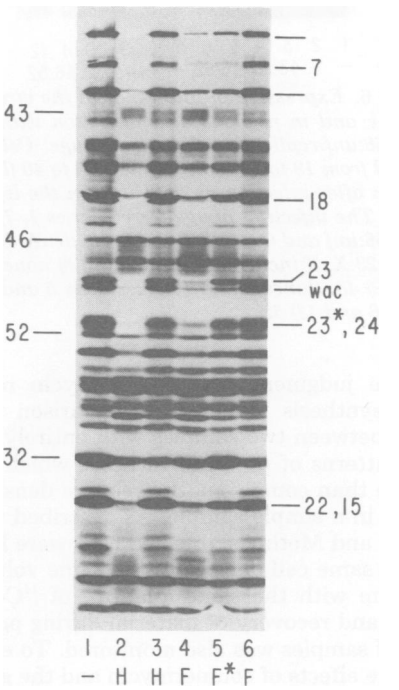

FIG. 5. *Expression of chromosomal genes and cloned gene 23 in the presence of metabolic inhibitors of replication. E. coli B834S(pVH503) was infected with denA:denB:12:23 phage. Five minutes (lanes 2, 4 and 5) or 25 min (lanes 3 and 6) later, portions were transferred to hydroxyurea (H) or FUdR and uracil (F). At 33 min after infection, one-half of the 5-min FUdR-treated sample was transferred to deoxyadenosine and thymidine (F*). All samples were labeled from 34 to 40 min after infection. Final concentrations of added substances were: (H) hydroxyurea, 13.7 mg/ml; (F) FUdR and uracil, 45 and 180 μg/ml, respectively; (F*) FUdR, uracil, thymidine, and deoxyadenosine, 45, 180, 350, and 40 μg/ml, respectively.*

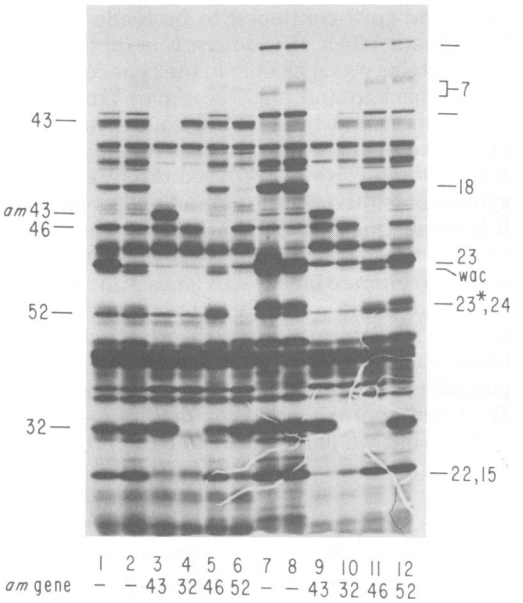

FIG. 6. *Expression of late genes in the viral chromosome and in pVH503 after infection with denA:denB:56:unf:replication-defective phage. Cells were labeled from 19 to 24 (lanes 1-6) or 35 to 40 (lanes 7-12) min after infection as described in the legend to Fig. 4. The infecting phage were: (lanes 1, 7) denA:denB:56:unf and (lanes 2-6 and 8-12) denA:denB:56:unf:12:23:X. X includes: (lanes 2 and 8) none; (lanes 3 and 9) 43; (lanes 4 and 10) 32; (lanes 5 and 11) 46; (lanes 6 and 12) 52.*

(The judgment that coumermycin reduced gp23 synthesis involves a comparison of one band between two samples with entirely different patterns of protein synthesis, which is less simple than comparing the relative densities of bands in a simple sample. As described in Materials and Methods, infected cells were labeled at the same cell density in the same volume of medium with the same quantity of ^{14}C-amino acids, and recovery of material during preparation of samples was also monitored. To emphasize the effects of coumermycin and the gene 52 mutation, different proportions of labeled samples were applied to the gel shown in Fig. 7. Lanes 1 and 2 contained approximately 1.7-fold more protein than lanes 3 and 4, and lane 6 contained approximately 2.4-fold more protein than lanes 5, 7, and 8.)

The effect that metabolic inhibitors of DNA replication exert on expression of chromosomal late genes and cloned gene 23 was also examined in the *denA:denB*:56:*unf* genetic background. Hydroxyurea was added 5 min before infection and 2, 10, or 20 min after infection, respectively, to bacteria carrying plasmid pVH503, and proteins were labeled 30 to 40 min postinfection (Fig. 8). The synthesis of the plasmid-coded gp23 was seen to be differentially resistant to inhibition by hydroxyurea relative to the other, chromosome-coded, late proteins. Comparing with Fig. 6 (lanes 9 and 10) suggested that metabolic inhibition of DNA replication affected gp23 synthesis less than did mutational inactivation of the gp43 and gp32 replication proteins. This stands in contrast with what was observed in the *denA:denB* genetic background (Fig. 4 and 5), where the cloned gene 23 and the other late genes responded comparably to inhibition of DNA replication.

We further examined two aspects of replication-independent gp23 synthesis: its time course and its regulation by the gene 55 protein. Figure 9 shows the kinetics of synthesis after infection with 12:23 phage in the *denA:denB*:56:*unf* and *denA:denB*:56:*unf*:43 genetic backgrounds; Fig.

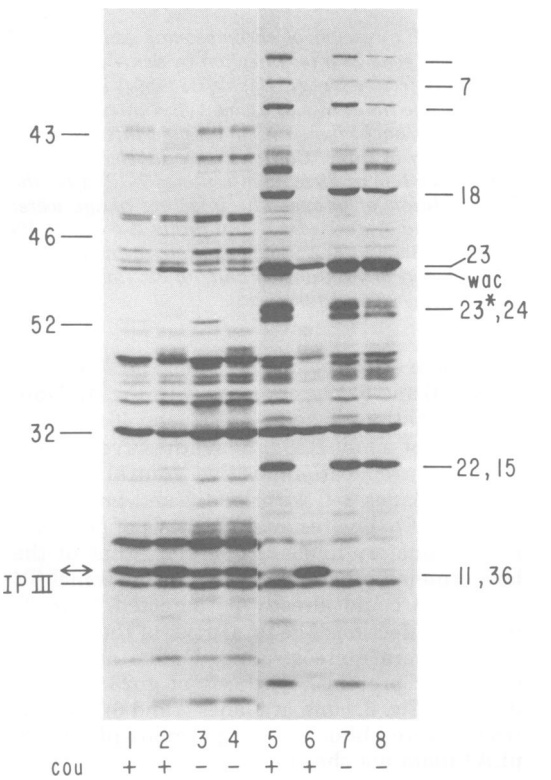

FIG. 7. *Effect of coumermycin on expression of chromosomal genes and of cloned gene 23. E. coli B834S(pLA1) was infected with denA:denB:56:unf:12:23:52 (even lanes) or denA:denB:56:unf:12:23 phage (odd lanes) and labeled 9 to 14 (lanes 1-4) or 33 to 38 (lanes 5-8) min after infection. Coumermycin (250 μg/ml) was added to some of the cells 10 min before infection (lanes 1, 2, 5, and 6).*

3 presents the comparable experiment on the *denA:denB* genetic background. Figure 9 shows progressively increasing rates of gp23 synthesis, both in the 43^+ and in the 43^- infection. Surprisingly, gp23 (or a protein with the mobility of gp23) appears to be made continuously throughout infection (although only at a low level, when examined from 10 to 15 min after infection). This contrasts with the *denA:denB* experiments, where synthesis of gp23 was not detected at any time during *denA:denB*:43 infection (Fig. 3b).

To further analyze the apparently precocious synthesis of gp23, cells harboring pLA1 or pBR322 were infected with *denA:denB*:56:*unf*: 12:23:55 phage. The comparison showed that in both kinds of cells, in which host protein synthesis is shut off rather slowly, a host protein with the mobility of gp23 was synthesized up to 10 min after infection (data not shown). Thus, the protein with the mobility of gp23 in Fig. 9 (lanes 1 and 6) was not necessarily gp23. A more decisive result came from an experiment in

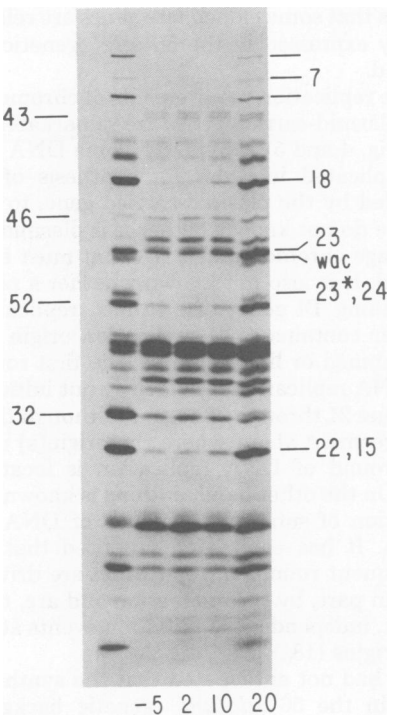

FIG. 8. *Expression of chromosomal genes and of cloned gene 23 in the presence of a metabolic inhibitor of DNA replication. E. coli B834S(pVH503) was infected with denA:denB:56:unf:12:23 phage. Cells were transferred to hydroxyurea (final concentration, 13.7 mg/ml) 5 min before or 2, 10, or 20 min after infection (lanes 2-5, respectively). The control samples (lane 1) had no hydroxyurea. Proteins were labeled from 30 to 40 min after infection.*

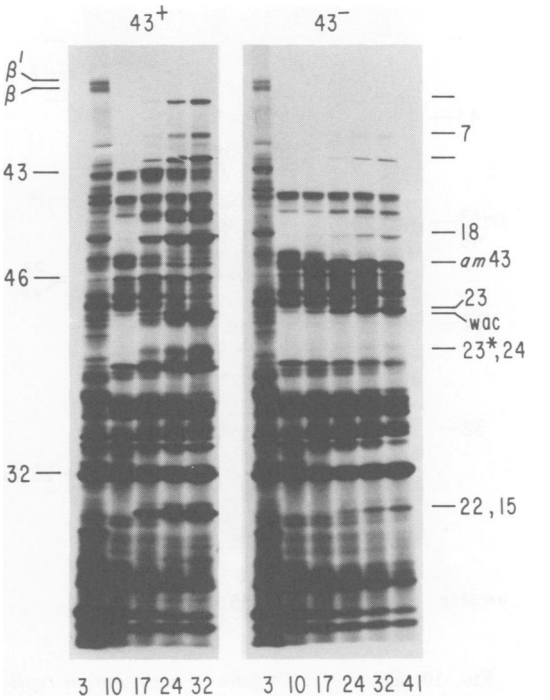

FIG. 9. *Kinetics of synthesis of T4 late proteins. Contributions from the viral chromosomes and from pVH503 after infection with denA:denB:56:unf:12:23: $43^{+/-}$ phage. Cells were infected and pulse-labeled for 5 min as described in the legend to Fig. 3. (Left) 43^+; (right) 43.*

which replication-independent and plasmid-determined gp23 synthesis was examined for its dependence on gp55 (Fig. 10). Plasmid pLA4, which lacks a gene 21 to 22 segment that is present in pVH503, was used here, but similar results were obtained with pVH503 and pLA1. pLA4 complements gene 23 mutants very effectively and also shows strong negative complementation (10). *E. coli* B834S(pLA4) was infected with gene 43, 32, 46, or 52 mutant derivatives of *denA:denB*:56:*unf*:12:23 phage and labeled 34 to 41 min later, when host protein synthesis had long since stopped. The rate of plasmid-dependent gp23 synthesis in the control DNA replication-positive phage infection (lane 1) was very high. As already noted, it was apparently unaffected by the gene 52 mutation (lane 5), reduced somewhat by a gene 46 mutation (lane 4), and possibly reduced slightly more by gene 43 and 32 (DO) mutations (lanes 2 and 3). Additional gene 55 mutations reduced all of this gp23 synthesis to a very low but clearly detectable level (compare lanes 6–10 with lane 1) which was more or less the same for the five genotypes being tested, showing that it was rep-

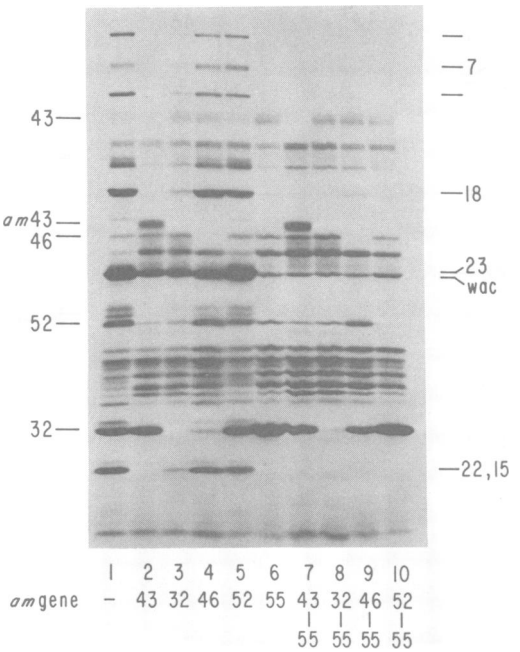

FIG. 10. *Effect of a T4 gene 55 mutation on replication-independent expression of cloned gene 23. E. coli B834S(pLA4) was infected as described in the legend to Fig. 3 and labeled from 34 to 41 min later. The infecting phage were denA:denB:56:unf:12:23 (lane 1) or the same phage with additional amber mutations in genes 43 (lane 2), 32 (lane 3), 46 (lane 4), 52 (lane 5), 55 (lane 6), 43 and 55 (lane 7), 32 and 55 (lane 8), 46 and 55 (lane 9), and 52 and 55 (lane 10).*

lication independent. Remembering that gp23 was made in uninfected pLA4 maxicells (Fig. 1a, lane 4), we suspect that there was, in fact, a continuous low level of synthesis of gp23 off the plasmid-borne gene 23. This continuation level was much lower than that afforded by replication-independent plasmid-coded gp23 synthesis (Fig. 10, lanes 2 and 3).

In summary, in a *denA:denB*:56:*alc/unf* genetic background, plasmid-borne gene 23 activity was sensitive to the presence of replication proteins (with the exception of the DNA topoisomerase partly coded by gene 52), but not in the absolute sense seen in the *denA:denB*:56^+:alc^+ genetic background. Synthesis of the plasmid-coded gp23 was somewhat sensitive to hydroxyurea, which blocked DNA replication, but not in the absolute sense seen in the *denA:denB*:56^+:alc^+ genetic background. The replication-independent synthesis of gp23 programmed by the plasmid was largely dependent on the gp55-containing form of T4-modified RNA polymerase. We suggest (but do not prove) that the cloned gene 23 is expressed at a low rate in uninfected cells. This expression is turned off by *denA:denB*:56^+:(alc^+) but probably not (entirely) by *denA:denB*:56:*alc/unf* phage.

DISCUSSION

In this paper, we have examined the regulation of the expression of the cloned gene 23 as a means of understanding the mechanistic basis of complementation and, ultimately, of T4 late gene expression in general. The two different genetic backgrounds used for this analysis of protein synthesis yielded rather different results, which are commented on separately. Finally, some proposals connecting these experiments are put forth.

The 56^+:*alc/unf*$^+$ case. The analysis of protein synthesis confirms what the complementation experiments have already told us: gp23 can be made in this genetic background. It is important, however, to remember that *alc* effects on complementation vary quantitatively from gene to gene (Table 10 in reference 10). This probably means that some cloned late genes are relatively poorly expressed in the 56^+:alc^+ genetic background.

The replication requirements of chromosomal and plasmid-carried genes are remarkably similar (Fig. 4 and 5). Obviously, some DNA has to be replicated in order for synthesis of gp23, encoded by the plasmid-carried gene, to occur, but we do not know whether it is plasmid DNA or phage chromosomal DNA that must be replicated. It is also not known whether a plasmid containing T4 genes can, in fact, replicate if it neither contains a T4 replication origin nor is recombined or linked to one: the first round of T4 DNA replication is evidently not initiated in the gene 21 through 23 region (although there is disagreement about where the origin[s] of that first round of DNA replication is located [6, 19]). On the other hand, nothing is known about initiation of subsequent rounds of DNA replication. It has even been proposed that these subsequent rounds of replication are driven, at least in part, by recombination and are, to that extent, independent of initiation events at replicon origins (18a).

We had not anticipated that the synthesis of gp23 in the 56^+:*alc/unf*$^+$ genetic background would depend almost absolutely on the state of gene 46. The effect of a gene 46 mutation signifies, in principle, either that activity of gene 23 on the plasmid directly requires gp46 or that lack of gp46 leads indirectly to a destabilization or destruction of that form of the plasmid which is competent to make gene 23 mRNA. Lacking specific evidence for indirect effects, considerations of parsimony require that we confine our-

selves to simpler models in which the involvement of gp46 is a direct one. At first glance, a gene 46 involvement implicates recombination. Yet we have shown in the preceding paper (10) that when one demands recombination in gene 23 as a prerequisite to complementation (by using plasmids with appropriate deletions): (i) only minimal complementation is generated in a 56^+:*alc*$^+$ genetic background; and (ii) although recombination may be capable of generating some complementation, it clearly is not required for complementation in a 56:*alc/unf* genetic background. Therefore, the relationship of gp46 to complementation is not the obvious one. It is not merely a matter of having to transfer the plasmid-borne gene 23 to the phage chromosome (e.g., to a late promoter on the phage chromosome) in order to have that late gene expressed. Regarding the DNA replication requirement, two alternatives deserve some consideration. The first is that the required replication takes place on the chromosome and that gp46 is required for using this replicating DNA to "activate" the plasmid for late transcription (perhaps via single-stranded DNA, generating structures that might resemble three-stranded recombination loops; cf. (8, 18a, 23). The second proposal is that replication of the plasmid is required simply in order to put hydroxmethycytosine into the plasmid-carried late gene, which then functions like the phage chromosomal late genes; gp46 may be required for plasmid DNA replication (for reasons that are not yet understood but might have to do with its exonuclease function or with recombinational activation of DNA replication; cf. 19, Mosig et al., in press). The first proposal raises the question of how gene 23 gets to be expressed in cytosine-containing DNA in an *alc*$^+$ cell.

T. Mattson, A. Bolle, G. van Houwe, and R. H. Epstein (personal communication, manuscript in preparation) have also found very strong effects of mutations in gene 46 and in gene 44 (the latter codes for another replication protein) on plasmid-coded gp23 synthesis. We should point out that, in disagreement with us, they interpret their experiments in terms of a literal recombination requirement for plasmid complementation.

The 56:*alc/unf* case. In the 56:*alc/unf* genetic background, the plasmid-borne gene 23 and the phage chromosomal late genes clearly are not regulated in the same way.

We have shown that the chromosomal late genes follow the same regulatory rules in the 56:*alc/unf* and 56^+:*unf*$^+$ genetic backgrounds, with respect to gene 55, DNA replication, and DNA replication delay caused by a gene 52 mutation. The plasmid gene 23 expression is also under gp55 control, although one does detect a small gp55-independent activity. However, this residue of activity is small relative to everything else we have discussed, and we shall not comment on it further.

Plasmid-carried gene 23 is much less sensitive than the chromosomal late genes to perturbations of DNA replication (generated by adding hydroxyurea to infected cells or by nonsense mutations in genes 43 and 32 and, in a recent experiment, gene 62). Similarly, a mutation in gene 52 delays chromosomal late gene expression, whereas, if the cell's DNA gyrase is functioning, expression of the cloned gene 23 is apparently independent of gp52. This creates the possibility of having the plasmid-coded gp23 made several minutes before the other late proteins can be detected (Fig. 6). If the cell's DNA gyrase is inhibited by coumermycin, the gp52-independent activity of the cloned gene 23 is reduced, but not as severely as is chromosomal late gene expression. Phage DNA replication is delayed by gene 52 mutations, and coumermycin blocks even this delayed replication (16). Since the inhibitory effect of coumermycin is exerted before the time at which gene 52 mutation-delayed chromosomal DNA replication starts, it seems likely that inhibiting both the host's DNA gyrase and the phage's type II topoisomerase directly and substantially inhibits expression of plasmid-borne gene 23.

The gene 46 mutation also exerts a substantial effect on gp23 synthesis in this genetic background, lowering the production of gp23 almost as much as do mutations in the replication genes 43 and 32.

What functions do the replication proteins and gp46 provide? There are indications that several of the replication proteins must, in some way, interact with late transcription proteins (21, 34). We also propose that gp46 drives some activation process for the plasmid-borne gene 23 in this genetic background. Replication should affect gp46-dependent activation; some of the replication dependence of gp23 synthesis in this genetic background might be due just to this interaction. The lack of effect of the gene 52 mutation, then, presents a problem, since chromosomal replication is here delayed by about 15 min, whereas gp23 synthesis is not detectably affected. There may be a balance of effects here, involving a compensatory increase of gp46-dependent activation by the gene 52 mutation (gene 52 defects are somewhat recombinogenic and lead to overproduction of gp32, perhaps because of the presence of excess single-stranded DNA; 7, 11). The fact that the activity of plasmid-borne gene 23 is partially independent of gp46 could mean either that the gene 23 pro-

moter on the plasmid works to some extent without activation in the 56:*alc* genetic background or that the process of activation occurs (with a lower rate or to a lesser extent) in the absence of gp46 when cytosine-containing DNA is involved. Why is the expression of late genes on the phage chromosome more or less independent of gp46? Perhaps because the phage chromosome is complete, extending outside the small region that is cloned in the plasmid. Those outside regions perhaps contain sites at which some other activation process substitutes for the gp46-dependent activation.

In the *den*:56:*unf* genetic background, there is no need to propose that a recombination event provides a chromosomal promoter for the plasmid: at least with respect to gp52, the plasmid simply does not follow chromosomal rules. It is much more plausible to consider the plasmid as a separate transcription entity. In the phage-infected cell it certainly behaves, in terms of gp55 dependence (and, according to a recent preliminary experiment, strong, though incomplete, gp45 dependence), as though it contains a late polymerase transcription unit. The replication proteins are involved in the transcription quantitatively, but there is partially independence (another differential effect, relative to the chromosomal late genes).

Conclusion. The data presented in this paper strongly suggest that, for plasmid-carried gene 23 as for chromosomal late genes, promoter activation involves some structural alteration of DNA. For chromosomal genes, this poorly understood activation was previously called "competence." For the plasmid-borne gene 23, activation of a promoter(s) in cytosine-containing DNA is absolutely dependent on replication in the *denA*:*denB*:(56$^+$:*alc*$^+$/*unf*) genetic background. In the *denA*:*denB*:56:*alc* genetic background, activation is enhanced by, but not exclusively dependent on, DNA replication. It is conceivable that in this genetic background there is no absolute requirement for promoter activation. Alternatively, either some other, less efficient activation process may ensue or transcription of the plasmid-borne gene 23 may involve major and minor promoters with different properties. What is now required to understand this activation process is to subject actively transcribing T4 late genes to biochemical and structural analysis. We hope that the cloning of these genes makes that analysis accessible.

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