JOURNAL OF VIROLOGY, Oct. 1977, p. 28-40
Vol. 24, No. 1

Printed in U.S.A.

Properties of the Nonlethal Recombinational Repair x and y Mutants of Bacteriophage T4

II. DNA Synthesis

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Received for publication 10 February 1977

The bacteriophage T4 recombination-deficient mutants *x* and *y* exhibited decreased rates of DNA synthesis as compared to wild-type T4. Mutant-induced DNA synthesis was more sensitive to mitomycin C than was wild-type synthesis. However, DNA synthesis in mutant- and wild-type-infected cells exhibited the same sensitivity to UV light and X-irradiation. When high-specific-activity label was administered at various times postinfection, mutant DNA synthesis resembled that of wild type for 12 min, after which time mutant-induced incorporation was greatly decreased and sensitive to mitomycin C as compared to that of the wild type. Rifampin and chloramphenicol studies indicated that the gene products necessary for synthesis measured at 15 min postinfection, including those of x^+ and y^+, were transcribed within 2 min and translated within 8 min postinfection. Administration of chloramphenicol to mutant *x*- or mutant *y*-infected cells exactly 8 min postinfection, however, allowed for increased synthesis at 15 min that was sensitive to mitomycin C. Cells coinfected with $T4^+$ and T4*x* or T4*x* and T4*y* retained a reduced mutant-type synthesis, whereas cells coinfected with $T4^+$ and T4*y* exhibited a synthesis more closely resembling that of wild type.

The recombination-deficient mutants of bacteriophage T4 can be roughly categorized into two classes. Those in the first class (3, 4, 29) cannot synthesize a series of gene products that are essential for late T4 replication and exhibit a DNA arrest phenotype. Mutants in the second class are viable and exhibit, in addition to recombination deficiency, a defect in DNA repair. The latter group of mutants appears to be analogous to the *rec* mutants of *Escherichia coli*.

The first of the nonlethal recombination mutants of T4, T4*x*, was isolated by Harm (12). Later, Boyle and Symonds isolated T4*y* (6); Van den Ende and Symonds isolated T4 1206 (33), and, recently, Hamlett and Berger reported the existence of an additional, nonlethal, recombination-altered mutant, T4*w* (11). T4*x*, *y*, and *w* were selected on the basis of their UV sensitivity. Mutant 1206 was selected by virtue of its low segregation of haploids from heterozygotes. Further, by examining the UV sensitivity of double mutants, it was determined that T4*x*, *y*, 1206, *w*, and a DNA delay mutant in gene 58 function in the same repair pathway—the *x*-*y* pathway—which is distinct from the excision pathway (5, 11, 12). In contrast to the latter, the *x*-*y* pathway for repair of UV damages requires prior phage DNA synthesis, as demonstrated by the functional survival experiments of Maynard-Smith and Symonds (20).

In addition to UV sensitivity and recombination deficiency, T4*x* and *y* have been shown to be sensitive to γ rays (6) and X rays (34), and T4*x*, *y*, and *w* have been shown to be sensitive to alkylating agents (1, 11, 22, 25). DNA synthesis and burst size in T4*x*-infected cells are sensitive to mitomycin C (32). Also, the T4*x* gene product, as well as the T4*w* gene product, appears to function in the repair of heteroduplex DNA (2). The T4*x* gene product and host polynucleotide ligase act in conjunction during the repair of ionizing radiation lesions (34). T4*x*, *y*, and 1206 have also been shown to be involved in an error-prone repair pathway leading to mutation induction (10).

All of these observations implicate the *x*-*y* pathway as a postreplicative recombinational repair process similar in many respects to the *rec* pathway in *E. coli*. However, one cannot carry this analogy too far because the replicative processes of T4 and its host are fundamentally different. T4 recombination appears to be

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intimately involved in the replicative process, whereas that of *E. coli* is not. This is exemplified by the failure to find a totally recombination-deficient viable mutant of T4 and by the lack of complementation of the *recA* function by the x^+ gene product (22). Clearly, dispensing with the gene products of the *x-y* pathway does not appear to affect the viability of the phage, although it does hamper the efficiency of this pathway in dealing with damaged DNA. Apparently, however, the *x* and *y* gene products are involved in the normal developmental processes in T4, since the burst size is reduced and the latent period is delayed (6, 11, 12, 13). Certainly, if genetic recombination can be considered to be a normal aspect of T4 development, the recombination-deficient phenotype can reflect the involvement of the *x* and *y* gene products in the development of T4.

These considerations provided the incentive for the following study on the DNA synthetic capacity of T4*x*- or *y*-infected cells. The T4*x* mutant used in these studies, T4*px*, has been extensively backcrossed by Drake to T4B (7) and probably represents a single mutation. T4*y*, originally isolated by Boyle and Symonds (6), has been shown to contain a suppressor that alters the plaque morphology (11).

(This work was submitted by R.J.M. in partial fulfillment of the requirements for the Ph.D. degree from The City University of New York, New York City, 1977.)

MATERIALS AND METHODS

Bacteria and bacteriophage. *E. coli* B and T4$^+$ (T4D) were originally supplied by A. Doermann. T4*v*, derived from T4D, was obtained from W. Harm; T4*y*, derived from T4D, was obtained from J. Boyle; and T4*px*, originally isolated as T4*x* from T4D by W. Harm and extensively backcrossed to T4B by J. Drake, was obtained from J. Drake.

Chemicals. [*methyl*-^{3}H]thymidine (63.8 Ci/mmol) and deoxyadenosine 5'-[8-^{3}H]triphosphate (102 Ci/mmol) were purchased from ICN. Mitomycin C, rifampin, and chloramphenicol were purchased from Calbiochem, La Jolla, Calif., and solutions of the antibiotics were freshly prepared before each experiment. Concentrations of mitomycin C were determined by using an $E^{1\%}_{326\ nm}$ of 700. Mitomycin C experiments were performed under dim light.

Media. The bottom- and top-layer agar, H broth, and gel buffer used were described previously (17). The phages were diluted in a broth containing 5 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of NaCl, and 2.5 g of K_2HPO_4 per liter of water. Citrate broth was prepared from H broth by substituting sodium citrate for sodium chloride. Supplemented M-9 medium contained 100 ml of 10× M-9 salts, 40 ml of 20% (wt/vol) glucose, 10 ml of 0.1 M $MgSO_3$, 10 ml of 0.01 M $CaCl_2$, 50 ml of 20% (wt/vol) Difco Casamino Acids (Difco), and 790 ml of sterile water. 10× M-9 salts contained 60 g of Na_2HPO_4, 30 g of KH_2PO_4, 5 g of NaCl, and 10 g of NH_4Cl per liter of water.

Preparation of bacteria and bacteriophage. Overnight cultures of *E. coli* B were prepared by incubation at 37°C with aeration in H broth. Daily cultures were prepared by a 100-fold dilution of the overnight stationary-phase cultures into supplemented M9 medium. The bacteria were then incubated at 37°C with aeration until they reached a concentration of 2 × 10^8/ml.

Bacteriophage stocks were prepared by eluting confluent plates and subjecting the lysates to differential centrifugation. The phage stocks were stored in gel buffer. For some experiments, T4*px* was further purified by sucrose gradient sedimentation.

Irradiation. For UV irradiation, a 30-μl phage suspension in gel buffer (0.5 × 10^{11} to 1 × 10^{11}/ml) was irradiated by a General Electric 15-W germicidal lamp (G 1578) (General Electric Co., Schenectady, N.Y.), powered through a Sola constant-voltage regulator, and filtered through a quartz-glacial acetic acid filter (34). The dose rate determined, as described previously (34), was 0.35 J/m^2.

For X-irradiation, a 200-μl phage suspension in 5× citrate broth (1 × 10^{10} to 5 × 10^{10}/ml) was irradiated by a Picker X-ray source with a beryllium window. A setting of 60 kVp and 20 mA delivered a dose rate of ca. 90 krads/min. This was originally determined (34) by ferrous sulfate dosimetry.

Incorporation of labeled DNA precursors. Log-phase *E. coli* (0.2 ml) at a concentration of 4 × 10^8/ml were added to 4 μl of a solution containing 10^{11} phage per ml. All reaction mixes were brought to final 0.3-ml volumes with M9 medium. Labeling was carried out at final thymidine concentrations of 0.12 or 25 μg/ml. At low thymidine concentrations, phages were adsorbed at 37°C and transferred to 20°C 1 min before labeling (33 μCi of ^{3}H/0.12 μg of thymidine per ml or 33 μCi of ^{3}H/0.16 μg of thymidine per ml). In one case, the entire experiment was carried out at 20°C. For long-term labeling, [^{3}H]thymidine was added to a final concentration of 33 μCi of ^{3}H/25 μg of thymidine per ml or 33 μCi of ^{3}H/15.8 μg of thymidine per ml. After a 4-min adsorption period, incubation was continued at 37°C.

In each of the above experiments, 20-μl samples were collected on Whatman GFA filters. They were immediately precipitated, batchwise, in 10% trichloroacetic acid and washed, batchwise, with 5% trichloroacetic acid and then with 95% ethanol. After drying, samples were counted in toluene-based scintillation fluid.

In experiments involving rifampin, chloramphenicol, or mitomycin C, the drugs were added at the times and concentrations indicated in the text. The concentrations of rifampin and chloramphenicol were maximally inhibitory under the conditions in which they were used. [^{3}H]dATP incorporation in toluenized cells was achieved by using the procedure of Miller et al. (21).

RESULTS

DNA synthesis measured under conditions of linear incorporation. DNA synthesis in T4$^+$-, *x*-, and *y*-infected cells was measured by the

incorporation of tritiated thymidine into the acid-insoluble fraction. The linear rate of label incorporation was reduced in T4*x*- and *y*-infected cells to about 70% of that of wild type (Fig. 1A). Similar results were obtained for T4*x* with [^{3}H]thymine (Fig. 1B). We have consistently observed a 20 to 30% reduction in the DNA synthetic rate in the mutant-infected cells when compared to that in wild-type-infected cells.

It is not clear why Shimizu and Sekiguchi (32) did not observe a difference in DNA synthetic rates between T4$^+$ and T4*x*. There are, however, several differences between their experiments and ours, such as their use of a *polA* mutant under conditions where incorporation in uninfected cells was as great as in infected ones. Also, T4*x* rather than T4*px* was used in their measurements of label incorporation into DNA.

When incorporation of labeled triphosphate precursor was measured in T4-infected cells toluenized 15 min postinfection, little synthesis was observed with T4*x* (Fig. 1C) as compared to wild-type-infected cells. In a later section, we will show that this is a critical period in the mutant-infected cells, which may explain the dramatic difference observed here.

Because of the role of the *x-y* pathway in DNA repair, the DNA synthetic capacity of T4-infected cells was examined after the phage were UV- or X-irradiated. Both UV- and X-irradiation produced a lag and a reduced final rate of DNA synthesis in both wild-type- and mutant (including T4*v*)-infected cells (data not shown). When the final slopes of DNA synthesis curves obtained by measuring incorporation of [^{3}H]thymidine were compared at different doses, the results shown in Fig. 2A and B were obtained. The DNA synthetic capacity of T4*v* was UV-inactivated at a slightly greater rate than that of the wild type or T4*x*, whereas that of T4*y* was inactivated at a decreased rate. The DNA synthetic capacities of T4*x* and T4*y* appeared to be slightly less sensitive to inactivation by X-irradiation than was that of the wild type. Shimizu and Sekiguchi (32) observed that DNA synthesis in T4*x*-infected cells was sensitive to UV light. However, they were comparing DNA synthesis after irradiation to zero-dose T4*x* controls that showed no synthetic difference from wild-type cells. In contrast, in the present studies, the zero-dose controls already exhibited a 30% decreased synthetic rate compared to that of wild-type-infected cells. Thus, the discrepancy appears to lie in the measurement of DNA synthesis, not in relative UV sensitivity.

It might be expected that the kinetics of label incorporation into DNA after irradiation is complex. Without irradiation, replicative synthesis would include a recombinational component. After irradiation, excision repair synthesis should precede replicative synthesis, and the recombinational repair component of the latter might be expanded. The total measurement would include all three. Since *v*-induced excision repair appears to occur before replicative synthesis (26), one might expect the increased UV sensitivity of T4*v* DNA synthesis, as has been observed here and elsewhere (8). On the other hand, if excision repair is expanded in the absence of *x-y* recombinational repair, one might expect a decreased radiosensitivity of DNA synthesis for the *x* and *y* mutants, as was shown above.

Figure 2C shows the effect of mitomycin C on the DNA synthetic capacity of T4$^+$, T4*x*, and T4*y*. In contrast to the above experiments where the free phages were irradiated, phage-bacterium complexes were exposed to mitomycin C throughout infection. Although this drug

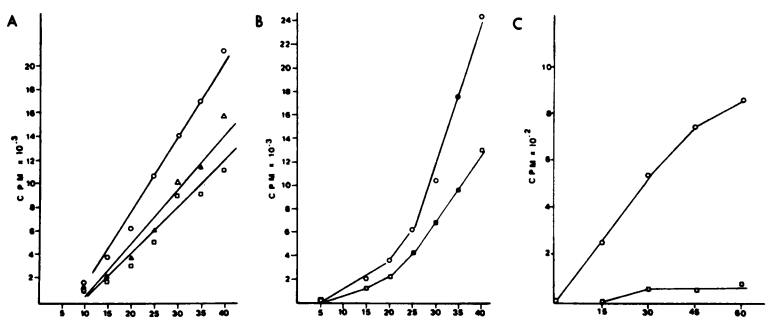

FIG. 1. *DNA synthesis in cells infected with T4$^+$ (○), T4x (□), and T4y (△) measured at 37°C. (A) [^{3}H]thymidine incorporation, 25 μg/15 μCi per ml. (B) [^{3}H]thymine incorporation, 25 μg/15 μCi per ml. (C) [^{3}H]dATP incorporation in toluenized cells.*

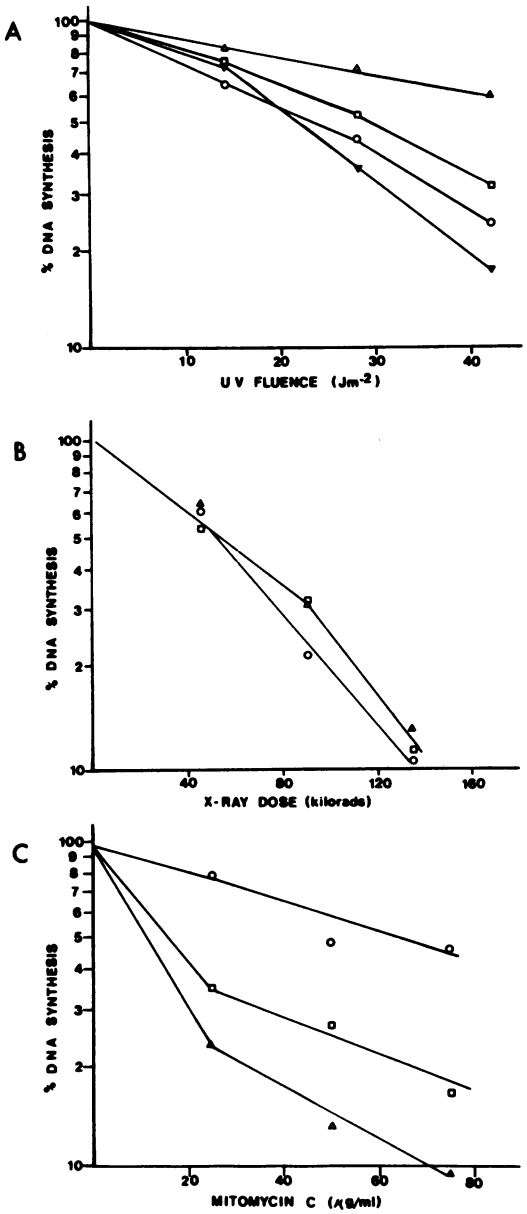

FIG. 2. *Inactivation of thymidine incorporation in cells infected with T4⁺ (○), T4x (□), T4y (△), and T4v (▽) at 37°C as calculated from the final slopes of DNA synthesis curves. (A) UV inactivation of free phage. (B) X-ray inactivation of free phage. (C) Mitomycin C inactivation of phage-bacterium complexes.*

affected wild-type DNA synthesis at higher doses, it was a more potent inhibitor of T4x and y DNA synthesis. T4x and y were also more sensitive to mitomycin C when viability was measured (Fig. 3). Shimizu and Sekiguchi (32) have shown T4x DNA synthesis and average burst size to be sensitive to mitomycin C. It is interesting that the original studies of Sekiguchi (27), which compared the sensitivity of DNA synthesis to mitomycin C in uninfected and T4-infected cells, showed that, although DNA synthesis in T4-infected cells was more resistant to mitomycin C than was host DNA synthesis, the resulting phages were not viable.

DNA synthesis measured by high-specific-activity pulses. To more clearly pinpoint the alteration in DNA synthesis in x- and y-infected cells, high-specific-activity thymidine was administered at various times postinfection, and the acid-insoluble fraction was measured. Cells were infected at 37°C, placed at 20°C 1 min before adding [³H]thymidine, and then sampled every 15 s (Fig. 4). Label incorporation proceeded at the same rate in T4⁺-, x-, and y-infected cells until 12 min postinfection, at which time incorporation in the mutant-infected cells decreased dramatically. This phenomenon was more closely examined in cells infected and maintained at 20°C. For example, label incorporation at 20°C in wild-type-infected cells continued to increase until 60 min postinfection, whereas in T4x- and T4y-infected cells, incorporation increased until 45 min and then decreased at 60 min (Fig. 5). Tritiated thymidine (65 Ci/mmol, 0.12 μg/ml, 37°C) was incorporated in T4⁺-, T4x-, and T4y-infected cells that were either amber suppressor positive (*E. coli* CR63) or amber suppressor negative (*E. coli* B; Fig. 6). Clearly, the amber y phenotype was suppressed in *E. coli* CR63, whereas T4⁺ and T4x were unaffected.

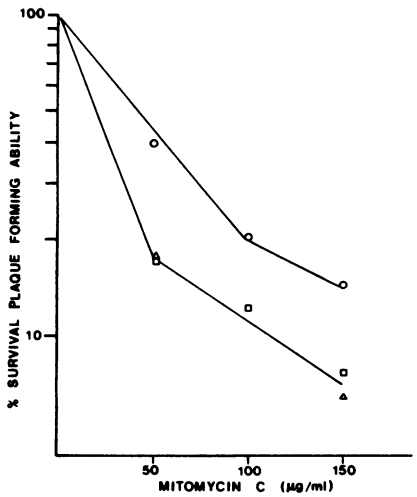

FIG. 3. *Mitomycin C inactivation of phage-infected cells after a 10-min exposure at 37°C of complexes to the drug before plating at 37°C. Symbols: T4⁺ (○), T4x (□), and T4y (△).*

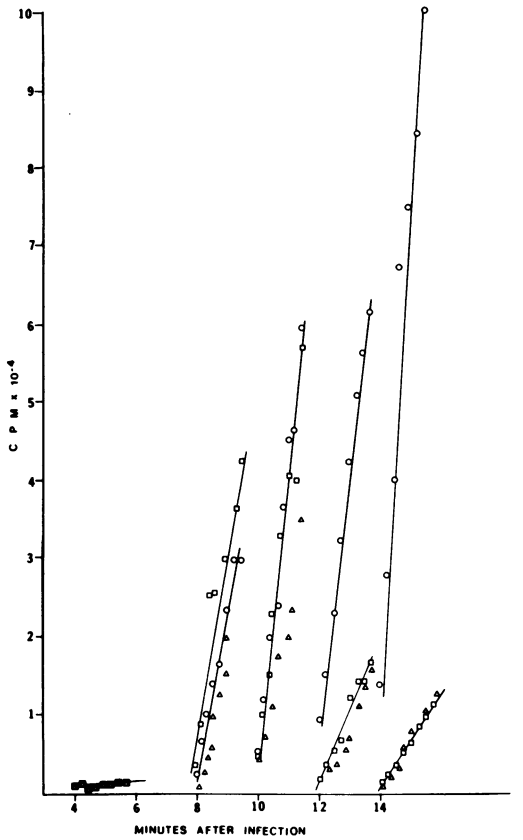

FIG. 4. *DNA synthesis at 20°C as measured by incorporation of high-specific-activity thymidine (65 Ci/mmol) in the absence of additional cold thymidine (final concentration, 0.12 μg/ml). Cells were infected with T4⁺ (○), T4x (□), and T4y (△) at 37°C and transferred to 20°C 1 min before labeling.*

The large difference in incorporation of high-specific-activity label between wild-type- and mutant-infected cells 15 min postinfection at 37°C does not appear to reflect a difference in the amount of intracellular label but does so in the amount of incorporated label (Fig. 7A). In these experiments, total cellular counts were measured by filtering the complexes through membranes (Millipore Corp., Bedford, Mass.), and acid-insoluble counts were determined as in Materials and Methods in the usual manner. The difference between these two numbers should reflect the amount of soluble label. The latter amounts were approximately the same for T4⁺-, *x*-, and *y*-infected cells.

Apparently wild-type-infected cells incorporated labeled thymidine very efficiently as compared to *x*-infected cells at the high specific activity (low thymidine concentrations) used in these experiments. When the labeled thymidine was diluted by cold thymidine, in a manner that more closely resembles conditions used for linear synthesis, the difference between wild-type and *x* incorporation was less pronounced (Fig. 7B). Dilution of labeled thymidine with cold deoxyadenosine showed the same effect. Also, dilution of labeled deoxyadenosine with cold thymidine reduced the incorporation of label in both wild-type- and mutant-infected cells (manuscript in preparation). Clearly, nucleotide pools during T4 replication are dynamic and complicated. Yegian et al. (39) have suggested that the DNA delay mutants that manifest all the normal DNA intermediates have alterations in their intracellular nucleotide pools. It appears, however, that the

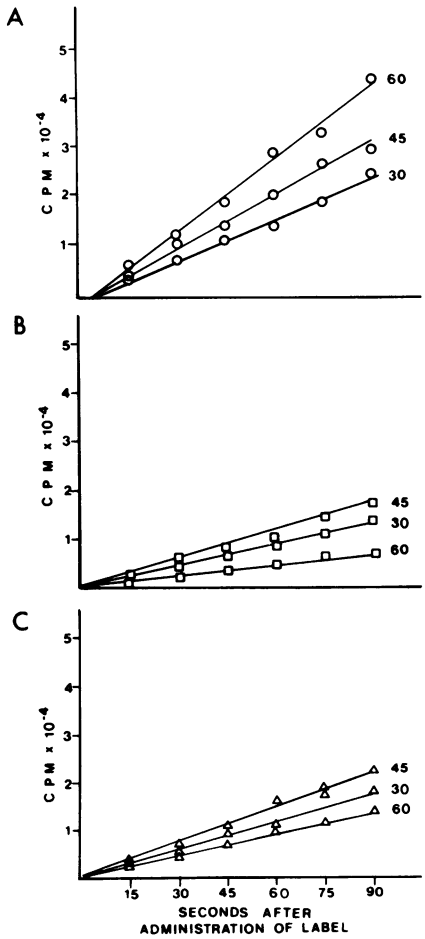

FIG. 5. *DNA synthesis at 20°C as measured by the incorporation of high-specific-activity thymidine (65 Ci/mmol) in the absence of additional cold thymidine (final concentration, 0.12 μg/ml). Cells were infected and labeled at 20°C at the times (in minutes) indicated. (A) T4⁺, (B) T4x, and (C) T4y.*

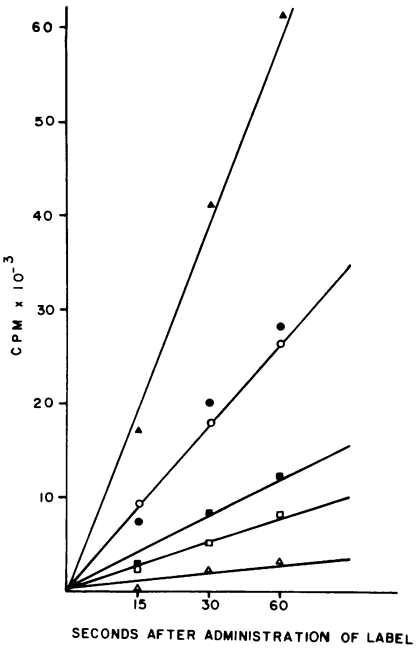

FIG. 6. *Effect of amber suppressor E. coli CR63 on thymidine incorporation. E. coli B or CR63 was infected with T4⁺, T4x, or T4y at 37°C. At 14 min postinfection, cells were transferred to 20°C for 1 min before labeling. Cells were labeled by using final concentrations of 0.12 μg of [³H]thymidine per ml (63 Ci/mmol). Open symbols: E. coli B; closed symbols: E. coli CR63. T4⁺ (○, ●); T4x (□, ■), and T4y (△, ▲).*

difference in rate of incorporation of high-specific-activity label between wild-type- and *x*- or *y*- infected cells reflects some difference in replication, since total label incorporation levels off at the same time in both cases; also, both are capable of taking up additional label, indicating similarity in thymidine processing in the pools (Fig. 7C and D). If there were a slower rate of uptake into the precursor pools with *x*, one might expect continued incorporation, albeit at a slower rate.

Effect of rifampin and chloramphenicol on the manifestation of the T4x and *y* gene products. In an attempt to delineate the transcription time of the *x* and *y* gene products, rifampin was administered at various times postinfection at 37°C, and subsequent incorporation of high-specific-activity thymidine was measured at 15-s intervals after administration at 15 min postinfection. Label incorporation by wild-type-infected cells was the same when no rifampin was added and when rifampin was added 4 min postinfection (Fig. 8). Since the *x* and *y* proteins are necessary for normal DNA synthesis later in infection, the T4*x* and *y* genes must be transcribed before 4 min postinfection. In fact, a comparison of DNA synthesis between mutant- and wild-type-infected cells indicates that the *x* and *y* transcripts were made even before 2 min. Interestingly, mutant-induced incorporation after rifampin addition did not resume its normal, low level of incorporation until 8 min postinfection. Thus, it is possible that genes essential for mutant DNA synthesis are not transcribed until later in infection, between 6 and 10 min.

To elucidate translational events, similar experiments were performed with chloramphenicol added at 4, 8, or 12 min postinfection, and label incorporation was measured after addition at 15 min postinfection. Chloramphenicol added at 4 min postinfection completely inhibited incorporation at 15 min in wild-type-infected cells, whereas chloramphenicol added at 8 or 12 min had little or no effect on it (Fig. 9A). Since this 15-min incorporation was a measure of the functioning of the *x* and *y* gene products (among others), these data indicate that the *x* and *y* transcripts were translated before 8 min. Likewise, adding chloramphenicol at 4 min postinfection completely inhibited *x*- or *y*-induced incorporation at 15 min. However, adding chloramphenicol at 8 min postinfection with T4*x* or *y* gave an incorporation level similar to that of the wild type, whereas incorporation by the mutant-infected cells in the absence of chloramphenicol was greatly reduced when compared to that of the wild type (Fig. 9B and C). This "rescue" was negligible or only slight when chloramphenicol was added 12 min postinfection. It appears that prevention of protein synthesis in T4*x*- or *y*-infected cells at a critical time—8 min postinfection—allowed for subsequent DNA synthesis that more closely equals that of the wild type, at least in quantity.

Effect of mitomycin C on incorporation of high-specific-activity thymidine. The effect of mitomycin C on incorporation of high-specific-activity thymidine was measured at 8 and 15 min postinfection. DNA synthesis at 8 min postinfection in wild-type- and mutant-infected cells was reduced to a similar small degree by mitomycin C (Fig. 10). In contrast, mutant-induced DNA synthesis at 15 min postinfection was more sensitive to mitomycin C than was that of the wild type. When chloramphenicol was added at 8 min postinfection and label incorporation was measured at 15 min, the increased "rescued" DNA synthesis observed in mutant-infected cells retained its 15-min sensitivity to mitomycin C (Fig. 11). Thus, even

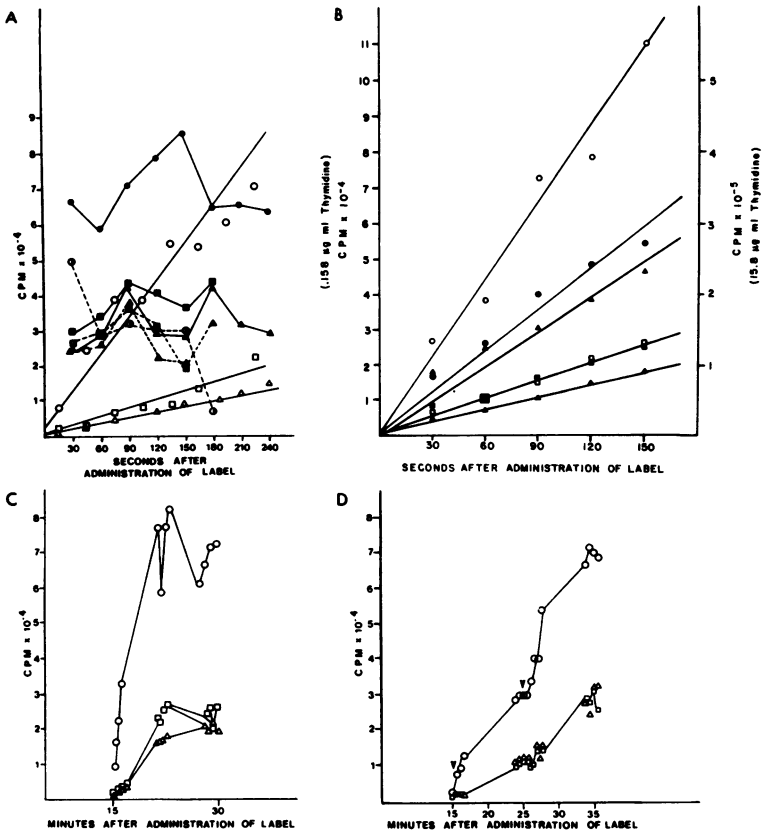

FIG. 7. *Parameters affecting apparent DNA synthetic rates. (A) Permeability of cells infected with T4⁺, T4x, and T4y to labeled thymidine as determined by measuring free intracellular label. Cells were infected for 14 min at 37°C. One minute after transfer to 20°C, they were labeled with 33 μCi of thymidine per ml (65 Ci/mmol). The final thymidine concentration was 0.12 μg/ml. Total intracellular label was determined by placing 20-μl portions onto Millipore filters and washing with 2 ml of M9 medium. Symbols: T4⁺ (●), T4x (■), and T4y (▲). From the same tubes at staggered 30-s intervals, incorporated counts were determined by collecting 20-μl samples onto GFA filters and precipitating with trichloroacetic acid. Symbols: T4⁺ (○), T4x (□), and T4y (△). Free intracellular label was determined by subtracting total intracellular label from incorporated label. Symbols: T4⁺ (◑), T4x (◨), and T4y (◭). (B) The effects of various thymidine concentrations on the incorporation of labeled thymidine into trichloroacetic acid-precipitable material were examined by labeling with 33 μCi of thymidine per ml in the presence of either a 0.158- or 15.8-μg/ml final concentration of thymidine. Counts for the latter were corrected for the difference in specific activity. Symbols: T4⁺ (○), T4x (□), and T4y (△), 0.158 μg of thymidine per ml; T4⁺ (●), T4x (■), and T4y (▲), 15.8 μg of thymidine per ml. (C) Cells were grown and infected as in (A), except that the label was followed for 15 min after its administration. Symbols: T4⁺ (○), T4x (□), and T4y (△). (D) Cells were grown and infected as in (A), except that an additional 33 μCi of label per ml was added 10 min after the addition of label at 15 min postinfection. Symbols: T4⁺ (○), T4x (□), and T4y (△).*

though the rate of DNA synthesis by the *x* and *y* mutants in the presence of chloramphenicol was similar to that of the wild type, it retained the mutant quality of mitomycin C sensitivity.

Complementation. In the following experiments, an attempt was made to measure gene complementation with respect to DNA synthesis. High-specific-activity thymidine was administered 15 min postinfection at 37°C, and incorporation into the acid-insoluble fraction was measured in cells infected with one or more genotypes. Coinfection with T4⁺ and T4*y* gave a wild-type phenotype, but coinfection with T4⁺ and T4*x* gave an intermediate phenotype (Fig. 12A). Coinfection with T4*x* and *y* showed little or no complementation. The *x* mutant used in these studies, T4*px*, has a point mutation (7) and could produce a protein that has partial function. For example, the mutant protein might still bind to the replicating complex and thus act as an inhibitor. This is supported by the observed inhibition of wild-type synthesis

in the presence of the x mutant and by the lack of significant complementation in T4x- and y-coinfected cells. Boyle and Symonds (6) were also not able to demonstrate significant complementation of x by x^+ in survival of phenotype experiments. Inhibition of DNA synthesis would not be as likely with T4y, since it is an amber mutant. It is possible that the x gene product is required stoichiometrically, and, therefore, the apparent inhibitory effect of the x mutant on wild-type DNA synthesis might be due to an insufficient amount of the x gene product.

Earlier experiments (Fig. 8) indicated that the x gene product was transcribed before 4 min. Thus, the addition of rifampin at 4 min to coinfected cells should distinguish between inhibitory products transcribed before and after this time (Fig. 12B). Cells coinfected with wild-type and x mutant phages exhibited an inhibition of DNA synthesis greater than that which was observed when coinfecting in the absence of rifampin. Coinfection of the wild type and y in the presence of rifampin exhibited a slight reduction in the wild-type level of synthesis. Also, coinfection with both x and y mutant phages did not give complementation. These experiments support the notion that the defective x gene product is transcribed before 4 min and is responsible for the inhibition observed.

It was unlikely that the increased mutant DNA synthesis observed in cells treated with chloramphenicol 8 min postinfection (Fig. 9) was caused by "defective x protein," since it also occurred with amber T4y. However, coinfection experiments were performed under these conditions as well. When chloramphenicol was added 8 min postinfection and synthesis was mea-

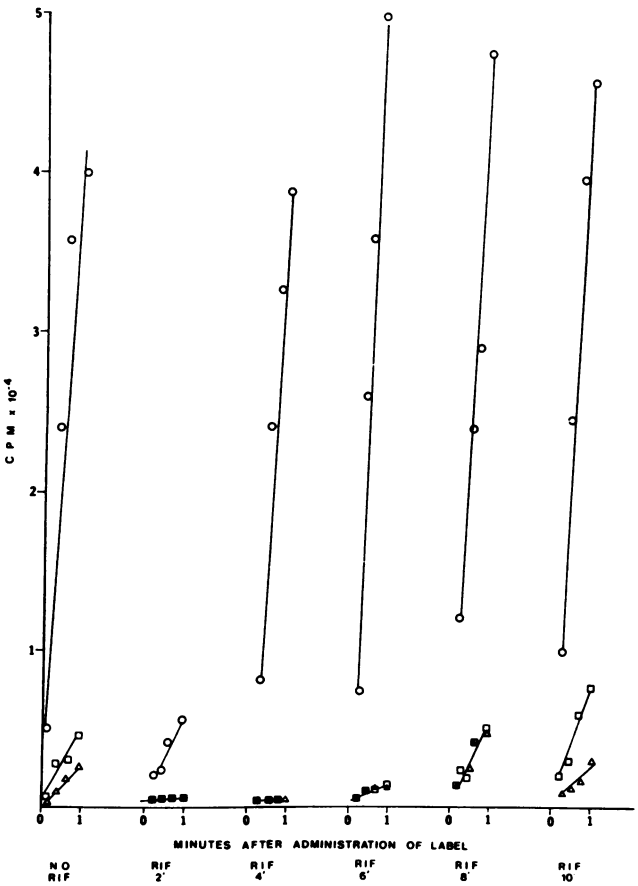

FIG. 8. *Effect of rifampin on DNA synthesis measured by high-specific-activity (65 Ci/mmol) incorporation of 33 μCi of thymidine per ml (final concentration, 0.12 μg/ml) at 20°C. The complexes were incubated at 37°C until 14 min postinfection, at which time they were switched to 20°C. Label was added at 15 min postinfection, and samples were taken at 15-s intervals thereafter. Rifampin (200 μg/ml, final concentration) was added at the indicated times after infection. Symbols: T4⁺ (○), T4x (□), and T4y (△).*

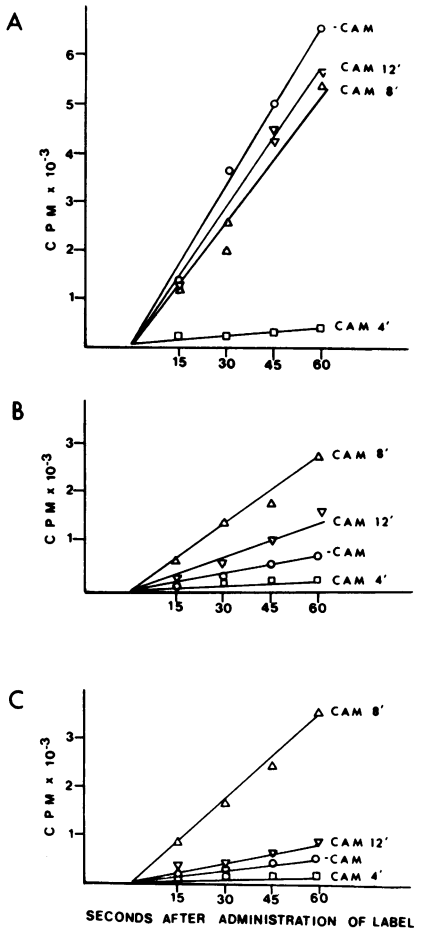

FIG. 9. *Effect of chloramphenicol on DNA synthesis as measured by incorporation of 33 μCi of high-specific-activity (65 Ci/mmol) thymidine per ml at a final concentration of 0.12 μg/ml at 20°C. The label was given 1 min after 14 min of postinfection incubation at 37°C. Chloramphenicol (200 μg/ml, final concentration) was added at the indicated times after infection. (A) T4⁺; (B) T4x; and (C) T4y. Symbols: No chloramphenicol (○); chloramphenicol, 4 min (□); chloramphenicol, 8 min (△); and chloramphenicol, 12 min (▽).*

sured at 15 min, coinfection of wild type with *x* gave an *x* phenotype, whereas coinfection with *y* gave a wild-type phenotype (Fig. 12C). Again, little or no complementation was observed with T4*x* plus T4*y*.

DISCUSSION

T4 has proven to be an extremely useful genetic tool, partly because of its high frequency of genetic recombination. Yet this same property also helps create a barrier to elucidating the T4 DNA synthetic events because of its intimate link with DNA synthesis. The existence of a class of partially recombination-deficient mutants may thus be the necessary tool to simplify the replicative process by minimizing the recombination-directed alteration of DNA. At

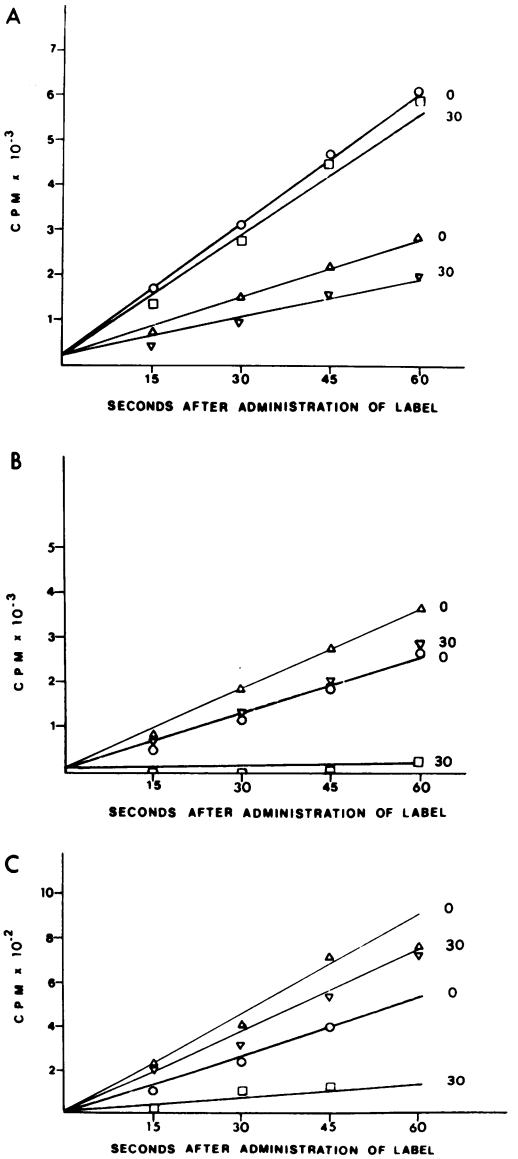

FIG. 10. *Effect of mitomycin C on DNA synthesis measured at 8 and 15 min after infection with T4⁺, T4x, and T4y. Infected cells were incubated at 37°C until 1 min before labeling, at which time they were switched to 20°C. Cells were labeled with 33 μCi (final concentration) of tritiated thymidine per ml (64 Ci/mmol). Mitomycin C was added to a final concentration of 30 μg/ml. (A) T4⁺; (B) T4x; and (C) T4y. Infected cells labeled at 8 min: minus mitomycin C (△); plus mitomycin C (▽). Infected cells labeled at 15 min: minus mitomycin C (○); plus mitomycin C (□).*

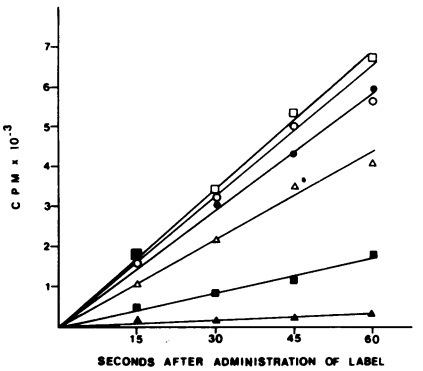

FIG. 11. *Effect of mitomycin C on chloramphenicol-rescued DNA synthesis. T4⁺-, T4x-, and T4y-infected cells were incubated at 37°C and treated with chloramphenicol (200 μg/ml, final concentration) at 8 min postinfection. One minute before labeling, cells were shifted to 20°C and [³H]thymidine (65 Ci/mmol) was added to 33 μCi/ml. Final mitomycin C concentration was 30 μg/ml. Symbols: T4⁺ (○); T4⁺ plus mitomycin C (●); T4x (□); T4x plus mitomycin C (■); T4y (△); and T4y plus mitomycin C (▲).*

the same time, by comparison to recombination-proficient phages, insights into the nature of recombination may be acquired.

The reduced rate of DNA synthesis found in T4x and T4y with high thymidine concentrations is in and of itself interesting, yet it cannot provide the sensitivity needed for more thorough investigations. The unexpected large difference in DNA synthesis that was observed between T4⁺ and the *x* and *y* mutants when labeling in low thymidine concentrations greatly increased the resolution and usefulness of simple labeling as an investigative probe. The impaired ability of the mutant-infected cells to incorporate thymidine did not appear before 12 min postinfection (Fig. 4). There appear to be two general explanations for this observation. The *x* and *y* mutant gene products may themselves inhibit the incorporation of DNA precursors. This possibility is indicated by the coinfection experiments in which the *x* genotype is found to inhibit wild-type DNA synthesis. Alternatively, the mutant DNA-defective phenotype may be a result of the missing gene products. For example, unless the *x* and *y* gene products function, the initiation of the events leading to *x-y* gene product-directed recombination may disrupt the replicating complex in such a manner as to reduce thymidine incorporation. In fact, the measurement of DNA synthesis under conditions of low thymidine concentration may be specific for examining recombination-directed DNA synthesis. Support for the latter alternative may be found in a recent paper by Wovcha et al. (35). They have found that there are apparently two independent pathways by which DNA precursors may become incorporated into DNA in T4-in-

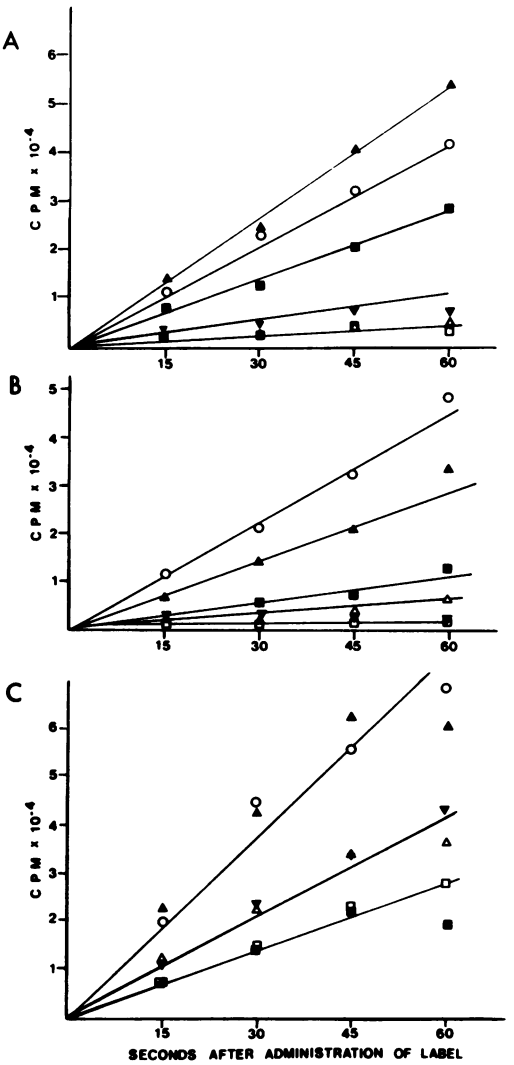

FIG. 12. *DNA synthesis in cells coinfected with mutant and wild-type phage. (A) Infected cells were grown for 14 min at 37°C and then transferred to 20°C for 1 min before adding 33 μCi of high-specific-activity (65 Ci/mol) thymidine per ml at a final concentration of 0.12 μg/ml. (B) Cells were infected and labeled as in (A) except that a 200-μg/ml final concentration of rifampin was added 4 min postinfection. (C) Cells were infected and labeled as in (A) except that a 200-μg/ml final concentration of chloramphenicol was added 8 min postinfection. The total input multiplicity of infection of phage was 10. Symbols: T4⁺ (○), T4x (□), T4y (△), T4⁺ plus T4x (■), T4⁺ plus T4y (▲), and T4x plus T4y (▼).*

fected cells. One pathway utilizes ribonucleotides, thymidine, and hydroxymethyl-deoxycytosine monophosphate. The second pathway requires the four deoxyribonucleoside triphosphates or monophosphates in addition to ATP. In the latter pathway, thymidine does not substitute for thymidine triphosphate. Both pathways require the standard T4-induced genes required for DNA synthesis such as T4 DNA polymerase. Work to determine the pathway(s) utilized by the *x* and *y* gene products is presently underway.

An alternative explanation that could account for the increased relative difference in thymidine incorporation by mutant- versus wild-type-infected cells, when measured under conditions of low-thymidine concentration, considers possible different specific activities of the DNA precursor pools. A recent paper by Flanegan and Greenberg (9) has shown that thymidine-containing DNA precursors in T4-infected cells do not appear to be controlled by feedback inhibition. They found that in T4 DNA mutant-infected cells that are unable to synthesize DNA, there is such an expansion of precursor pools that thymine and thymidine are excreted into the medium. Thus, if the relative rates of DNA synthesis measured in the presence of high concentrations of thymidine are, in fact, an accurate reflection of intracellular events, it might be expected than the 30% reduction synthesis found in *x* and *y* mutant-infected cells would lead to an accumulation of thymidine. This accumulation might, in fact, be large, relative to the exogenous thymidine that is added when synthesis is measured under pulse conditions. Therefore, a magnification of difference in the relative rates of DNA synthesis between *x* or *y* mutant- and wild-type-infected cells could be expected.

By adding rifampin at various times between 2 and 14 min postinfection and then measuring thymidine incorporation at 15 min, it is possible to further delineate some of the *x*- and *y*-related events that occur during T4 infection. There is a difference between mutant- and wild-type-induced DNA synthesis when rifampin is added as early as 2 min postinfection (Fig. 8). Thus, if the mutant-directed, DNA synthesis-deficient phenotype is a result of the absence of the *x* or *y* gene products, then the gene product(s) that acts on the altered substrate in their absence must also be transcribed within 2 min. Although there is a difference between wild-type and mutant-directed DNA synthesis measured at 15 min postinfection when rifampin has been added at 2 min, the levels of synthesis are well below those normally seen at 15 min. Therefore, the replication that occurs under these conditions is probably different from that which would occur under the normal sequences of transcriptional events. Similar reasoning may hold true when rifampin is added at 4, 6, 8, or 10 min postinfection, even though the rate of synthesis measured at 15 min appears more quantitatively normal.

Chloramphenicol may also be used to hold genetic expression at different times during the infection process. Adding chloramphenicol at 8 min postinfection did not impair the rate of DNA synthesis when measured 15 min postinfection in wild-type-infected cells. Adding chloramphenicol to mutant-infected cells at 8 min produced an apparent rescue of the mutant-directed DNA synthesis measured at 15 min postinfection. If the reduced levels of thymidine incorporation in non-chloramphenicol-treated, mutant-infected cells is a direct result of the mutant *x* or *y* gene products as discussed earlier, then chloramphenicol rescue may result from its preventing the mutant *x* or *y* gene products from exerting their effect. Alternatively, if the reduced level of synthesis found in mutant-infected cells at 15 min postinfection is due to the absence of wild-type *x* or *y* gene products, then chloramphenicol rescue might result from the prevention of the synthesis of proteins that alter the mutant-replicating DNA so that later levels of DNA synthesis are reduced. When considering the data from Fig. 11, which deals with the examination of mitomycin C sensitivity of DNA synthesis in cells treated with chloramphenicol at 8 min postinfection, the latter alternative seems more likely. The mitomycin C sensitivity of DNA synthesis is independent of the reduction in DNA synthesis found in T4*x*- or *y*-infected cells. To reiterate, chloramphenicol administered 8 min postinfection impedes development after the expression of the *x* and *y* gene products but before the expression of the protein(s) that is (are) responsible for reducing the mutant-directed levels of thymidine incorporation. Thus, mutant-infected cells, trapped at this stage of development, exhibited sensitivity of DNA synthesis to mitomycin C, whereas wild-type-infected cells, with functional *x* and *y* gene products, retained their relative resistance. This phenomenon is analogous to what is found in the recombination-deficient, DNA arrest mutants of T4 after chloramphenicol rescue of arrested synthesis. The DNA arrest phenotype of genes 46, 47, and 59 is reversed by adding chloramphenicol between 6 and 13 min postinfection or by the simultaneous presence of an additional mutation in genes 33, 55, *das*, or *dar* (14, 15, 16, 24, 28, 29, 30, 31, 36, 38). However, under some rescue conditions, there is no re-

versal of other phenotypes characteristic of the DNA arrest mutants. For example, there is no restoration of fast-sedimenting 200S DNA replication complexes (29, 30) or reversal of UV sensitivity and recombination deficiency (28, 37).

The nonlethal (x and y) and the lethal (DNA arrest) recombination-deficient mutants have altered rates of DNA synthesis at about the same time postinfection. This similarity suggests that the 46, 47, and 59 gene products may function in the same pathway as the x and y gene products. We are presently constructing double mutants between these two classes to examine this possibility. The effects of additional extragenic suppressor mutants on the x and y phenotypes will also be examined.

Although little is known about the mechanisms involved in T4 recombination, it does seem to occur subsequent to 12 min postinfection. The DNA arrest mutants stop synthesizing DNA at about this time, whereas the DNA delay mutants show increased rates of synthesis (19, 23, 39). Interestingly, the DNA arrest mutants exhibit decreased recombination, whereas the DNA delay mutants exhibit increased recombination (4, 18). It appears that the increased rates of synthesis found early in wild-type T4 infection (up to 12 min) reflect an increase in the number of replicating forks (19). In contrast, DNA replication measured late in infection probably represents DNA synthesis generated by recombination. The results of the thymidine incorporation studies presented in this paper suggest that the latter type of synthesis is greatly reduced in the x or y mutants and may be specifically measured by labeling with thymidine under conditions of low final thymidine concentration. Unlike the DNA arrest gene products, the x and y gene products had relatively little effect on the overall rate of DNA synthesis.

Many of the conditions that have been used in these studies to examine thymidine incorporation have also been used to examine sedimentation patterns in $T4^+$-, x-, and y-infected cells. The results of these experiments (manuscript in preparation) agree with those presented in this paper.

ACKNOWLEDGMENTS

We thank Sheila Jacobs for performing the toluenized-cell experiments and for helpful discussion.

This investigation was supported by Public Health Service research grant CA12693 from the National Cancer Institute and by a grant from the Research Foundation of the City University of New York.

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