Biochemical Studies on the x Mutation of Bacteriophage T4: Differential Inhibition of x^+ and x DNA Synthesis by Mitomycin C

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Biochemical studies were carried out to determine the effect of x mutation on T4 DNA synthesis. The rate and final extent of DNA synthesis are almost the same with T4D- and T4x-infected cells, although the burst size of T4x is about one-sixth that of the wild type. The DNA synthesis of T4x-infected cells is more readily inhibited by mitomycin C than is that of T4 wild type. When mitomycin C was added during active phage growth, DNA synthesis of T4x halted almost immediately. T4 DNA polymerases isolated from x^+ - and x -infected cells, however, exhibit no difference with regard to their sensitivities to mitomycin C, priming activities with alkylated or ultraviolet light-irradiated templates and other enzymatic properties.

Several genes of bacteriophage T4 are known to increase survival of phage after UV irradiation (4, 12). Gene v has been shown to control the formation of T4 endonuclease V, which catalyzes the initial incision step in excision repair of DNA (9, 30). Gene x , genetically distinct from v , appears to be involved in a reactivation mechanism different from excision repair (12, 13). Although the third gene, y , has been shown to affect radiation sensitivity in a similar manner to x (4), recent studies revealed that it is, unlike v and x , an essential gene for T4 growth (25). Recently, another T4 mutant, 1206, which also exhibits increased sensitivity to UV irradiation has been described (28).

Many phenotypic characteristics of x mutant have been reported, which suggest that x functions quite differently from v . The x mutant is sensitive to not only UV but also x - and γ -rays, alkylating agents and photodynamic action, and exhibits reduced ability of genetic recombination and slower rate of intracellular phage development (2, 4, 8, 10, 12, 13, 21). Genetic studies indicated that the diverse phenotype of T4x is attributed to a single mutation, which maps in the region where the genes for DNA synthesis cluster (7, 30). Consequently, one can assume that the pleiotropic phenotype of x mutation may be due to an alteration in one of the fundamental mechanisms involved in phage reproduction. Nevertheless, up to the present, little biochemical analysis of the T4x mutation has been made.

The present studies demonstrate that the DNA synthesis of T4x-infected cells is more readily inhibited by an antibiotic, mitomycin C (MC), than is that of T4 wild type, suggesting that the x mutation affects the DNA-synthesizing system of infected cells. An attempt was therefore made to compare DNA polymerases from the two types of infected cells.

MATERIALS AND METHODS

Bacterial and phage strains. *Escherichia coli* B was provided by S. Benzer. *E. coli* P3478 (*polA⁻ thy⁻*) was obtained from J. Cairns, and P3478R, a spontaneous revertant strain of P3478, was selected by M. Katsuki in this laboratory. Bacteriophage T4D (wild type) was provided by R. Edgar. T4v₁, T4x, and T4v₂ were furnished by W. Harm. T4px, a segregant from T4x (7), was provided by J. Drake.

Chemicals. The crystalline mitomycin C was a gift from N. Otsuji of Kyushu University. Carrier-free ³²P-orthophosphate was purchased from the Japan Radioisotope Association. ¹⁴C-thymine was obtained from the Daiichi Pure Chemicals Co., Ltd. ³H-dTTP and four types of unlabeled deoxyribonucleoside triphosphates were obtained from the Radiochemical Centre (England) and from the Schwarz BioResearch, respectively. Purified salmon sperm DNA was purchased from the Sigma Chemical Co. Methylmethane sulfonate (MMS) was obtained from the Tokyo Kasei-Kogyo Co., Ltd.

Incorporation of labeled compounds. Cell suspension, labeled with ³²P during active phage growth, was acidified by trichloroacetic acid and fractionated into DNA, RNA, acid-soluble nucleotide and phospholipid as described previously (23).

In the determination of incorporation of ^{14}C -thymine into DNA the cell suspension was acidified by trichloroacetic acid and collected on a membrane filter, and the radioactivity was determined in a liquid scintillation counter.

Assay and purification of T4 DNA polymerase. *E. coli* P3478 cells were infected with phage at a multiplicity of 6. After 30 min of infection, cells were chilled and harvested. Packed 26 g of infected cells were suspended in 100 ml of 0.05 M glycylglycine buffer (pH 7.0)-2 mM EDTA-2 mM reduced glutathione, and three 40-ml portions were sonically treated for 3 min each by a 20-kc ultrasonic disintegrator (Kaijyo Electrical Machinery Co., Ltd.). Unbroken cells were removed by centrifugation at $10,000 \times g$ for 20 min, and the supernatant fraction was taken as cell-free extract.

The procedures for assay and purification of T4 DNA polymerase were based on the methods of Goulian et al. (11). Incorporation of ^3H -dTTP into DNA was measured in a Packard Tri-Carb liquid scintillation counter.

To determine $3' \rightarrow 5'$ exonuclease activity in the DNA polymerase preparations, salmon sperm DNA, specifically labeled with ^3H -TMP at $3'$ -termini, was prepared by the limited reaction of T4 DNA polymerase. The exonucleolytic reaction was conducted in the following reaction mixture (0.3 ml): 67 mM Tris-chloride (pH 8.6), 6.7 mM MgCl_2 , 10 mM 2-mercaptoethanol, 16.6 mM ammonium sulfate, 6.7 μM EDTA, 0.33 mM NaF, 167 μg of bovine plasma albumin per ml, 0.24 mM $3'$ -terminally labeled ^3H -DNA (3×10^5 counts per min per μmol). Each enzyme (2.5 units) was added per tube and the reaction was performed at 37°C for 60 min.

Preparation of modified DNA. (i) UV irradiation of DNA. Heat-denatured salmon sperm DNA was dissolved in 0.02 M NaCl at a concentration of 200 $\mu\text{g}/\text{ml}$, and irradiated with a 15-W germicidal lamp (Toshiba) at room temperature. The dose rate was determined by a Black-ray J-255 short-wave UV intensity meter.

(ii) Alkylation of DNA. Heat-denatured salmon sperm DNA was incubated at 37°C in 0.02 M NaCl containing 0.01 M MMS. After incubation, the mixture was rapidly chilled and dialyzed at 2°C for 12 h against 1 liter of 0.01 M Tris-chloride (pH 7.5)-0.02 M NaCl.

Other methods. Deoxycytidylate deaminase activity was determined by a modification of the method of Keck et al. (16). DNA and protein were determined by the methods of Burton (5) and of Lowry et al. (17), respectively.

RESULTS

Nucleic acids synthesis in T4x-infected cells. The incorporation of ^{32}P -orthophosphate into several phosphate-containing compounds in T4D- and T4x-infected *E. coli* P3478 is shown in Fig. 1. Whereas phosphate metabolism of cells undergoes considerable change after infection with phage, there is no marked difference between cells infected with the wild type and x mutant. It seems that under the normal growth conditions the synthesis of

nucleic acids as well as of acid-soluble nucleotides and of phospholipids is not appreciably affected by the x mutation.

Different sensitivities of T4D and T4x DNA synthesis to mitomycin C. It has been demonstrated that the cellular and viral DNA synthesis have different sensitivities to an antibiotic, mitomycin C (18, 22). In an attempt to detect any alterations in the DNA-synthesizing system of T4x-infected cells, we have investigated the effect of MC on DNA synthesis. *E. coli* strain B was infected, at a multiplicity of 5, with T4D, x or v_1 and incubated for 60 min in the presence of various concentrations of MC. As shown in Fig. 2, the DNA synthesis of cells infected with phage is more resistant to the inhibitory action of MC than is that of uninfected cells, in agreement with the previous results (22).

Of much interest is the finding that there is a quantitative difference in susceptibility to MC between the T4 wild type and x mutant. DNA synthesis of T4x-infected cells is almost completely inhibited by 3 μg of MC per ml, whereas T4D-infected cells produce a considerable amount of DNA under the same conditions. This indicates that sensitivity to MC of DNA synthesis is controlled, at least in part, by the viral genome.

The pattern of inhibition of DNA synthesis of T4 v_1 -infected cells is almost the same as that of T4D-infected cells. It seems, therefore, that the v gene product, which functions to repair UV-induced damages in DNA, is not involved in the MC-inhibited process.

Mode of inhibition of T4x DNA synthesis by mitomycin C. The mode of inhibition of T4x DNA synthesis by MC was investigated in the next experiment, the result of which is shown in Fig. 3. MC was added to cells during active phage growth and the incorporation of ^{14}C -thymine into DNA was determined. In T4x-infected cells, the rate of incorporation decreased immediately after addition of MC and there is no net increase in radioactivity at 7 min after the addition. No such drastic effect of MC addition was observed with T4D-infected cells. From the result it may be suggested that MC inhibits the elongation step of T4 DNA replication when the normal x gene function is interrupted.

Although we have attempted to perform a similar experiment with *E. coli* P3478, the strain was found to be highly resistant to MC, and it was difficult to suppress the DNA synthesis even at 10 μg of MC per ml. This high resistance to MC of strain P3478 does not appear to be related with its deficiency in DNA polymerase I, since a revertant from P3478,

FIG. 1. Incorporation of ^{32}P by *E. coli* P3478 cells after infection. *E. coli* P3478 ($\text{pol A}^- \text{thy}^-$) was grown to 5×10^8 cells/ml at 37 C in 10 ml of phosphate-minimized Tris medium (pH 7.5) containing 0.1% Casamino Acids and 2 μg of thymine per ml. After addition of L-tryptophan, T4D or T4x was added to give a multiplicity of 5. After 5 min of infection, ^{32}P was added to give a final concentration of 1 $\mu\text{Ci/ml}$. At the times indicated, 1 ml-samples of the culture were transferred to tubes, which contain 1 ml of ice-cold 0.1 M sodium phosphate-10% trichloroacetic acid and 10^9 cells of unlabeled *E. coli* B. The suspension was fractionated into four phosphate-containing compounds as described previously (23). Symbols: $\times$, uninfected cells; $\circ$, T4D-infected cells; $\bullet$, T4x-infected cells. Incorporation of ^{32}P into (a) acid-soluble nucleotide, (b) phospholipid, (c) RNA, and (d) DNA.

which possesses normal level of polymerase activity, is also resistant to MC.

Effect of mitomycin C on the reproduction of phage. As a result of the preceding experiments it seemed important to study the effect of MC on the reproduction of T4x. It has been demonstrated that MC prevents the formation of infective phage, whereas it does not inactivate the infectivity of free phage particles (22).

E. coli B was singly infected with T4D, $\times$, px , or v_{11} , and incubated for 60 min in broth containing varying concentrations of MC. Strain px (pure $\times$) is a segregant from T4x and retains all the properties of the original $\times$

mutant except the *hm* (highly mutable) character (7).

The result is shown in Fig. 4 and indicates that the multiplication of phage is inhibited by relatively low concentrations of MC. There is no

FIG. 2. Effect of MC on DNA synthesis. *E. coli* B was grown to 5×10^8 cells/ml in broth, and infected with T4D, T4 v_{11} , or T4x at multiplicity of 4 and simultaneously various amounts of MC were added. The mixture was incubated at 37 C for 60 min and the amount of DNA was determined by diphenylamine reaction. As controls an uninfected culture and cultures infected by each of the three T4 strains were grown in the absence of MC. DNA synthesis in the MC-treated cells is presented as a percentage of synthesis in the untreated controls. Symbols: $\times$, uninfected; $\circ$, T4D-infected; $\bullet$, T4 v_{11} -infected; Δ , T4x-infected.

FIG. 3. Mode of inhibition of DNA synthesis by MC. *E. coli* B was grown to 5×10^8 cells/ml in M9 and infected with T4D or T4x at a multiplicity of 5. ^{14}C -thymine (a final concentration of 1 $\mu\text{Ci/ml}$) was added at 2 min after infection and MC was added at 20 min after infection to give a final concentration of 3 $\mu\text{g/ml}$. Samples of 0.5 ml were withdrawn at the indicated times and the radioactivity in the acid-insoluble fraction was determined. Symbols: $\circ$, T4D; $\bullet$, T4x.

FIG. 4. Effect of MC on phage growth. Conditions were as described in Fig. 2 except that the multiplicity was reduced to 0.02. Burst sizes in the MC-treated culture are presented as a percentage of the burst sizes in the respective untreated cultures. Average burst sizes in the control were: T4D, 124; T4v₁, 110; T4x, 21; T4px, 23. Symbols: O, T4D; □, T4v₁; △, T4x; ▲, T4px.

increase in number of infective phage when 1 µg of MC per ml is present in the medium. Since a considerable amount of DNA is produced by even T4x-infected cells under these conditions (see Fig. 2), it seems that most of the DNA produced in the presence of MC is biologically inactive. From the figure it is also noted that the reproduction of *x* is more readily inhibited by MC than is the reproduction of T4D and v₁.

DNA polymerase of T4x-infected cells. Figure 5 shows the formation of DNA polymerase and deoxycytidylate deaminase in cells infected with v₁ and with v₁x. Essentially similar patterns of enzyme synthesis were observed with *x*⁺ and *x* phages. Thus T4 DNA polymerase, which is responsible for DNA synthesis in T4-infected cells, is formed normally in T4x-infected cells.

To compare enzymatic properties of *x*⁺ and *x* DNA polymerases, we have isolated the enzymes from T4D- and T4x-infected cells. The enzymes were purified up to 1,300- to 1,500-fold according to Goulian et al. (11). The chromatographic behavior of *x*⁺ and *x* enzymes

on phosphocellulose or DEAE-cellulose column was similar, and the yield and specific activity of purified enzyme were comparable for the two preparations.

The effect of MC on the DNA polymerase reaction was first investigated and the result is shown in Fig. 6. In contrast to *in vivo* DNA synthesis, the reaction is not significantly affected by MC and there is no differential inhibition by MC of the *x*⁺ and *x* DNA polymerase reaction *in vitro*.

In the next experiments, the two enzymes were compared for their abilities to use modified DNA as template. Since MC does not react with DNA *in vitro*, DNA was modified by a mono-functional alkylating agent, methylmethane sulfonate (MMS). It has been demonstrated that MMS inactivates transforming DNA and free phage particles *in vitro* (2, 26). Furthermore, we found that ability of T4x or px to produce DNA and progeny phage is more readily inactivated by MMS than is that of T4 wild type (data not shown). In the experiment, heat-denatured salmon sperm DNA was incubated at 37°C in 0.01 M MMS, dialyzed for 12 h, and then used as template for the DNA polymerase reaction. As shown in Table 1, the reaction is slightly inhibited when the treated DNA is

FIG. 5. Induction of T4 enzymes in *E. coli* P3478 infected with T4v₁ or T4v₁x. *E. coli* P3478 was grown to 5×10^8 cells/ml in 100 ml of broth and infected with T4v₁ or T4v₁x at a multiplicity of 10. At the indicated times, samples (11 ml) were withdrawn, chilled in ice, and centrifuged. Cells were suspended in 1 ml of 0.05 M Tris-chloride (pH 7.5)-2 mM reduced glutathione-2 mM EDTA, disrupted by ultrasonication for 1 min, and then a cell-free extract was obtained by centrifugation. Deoxycytidylate deaminase activity (nanomoles of deoxyuridylate produced in 30 min by 0.1 mg of protein): □, T4v₁; ■, T4v₁x. DNA polymerase activity (counts per minute $\times 10^{-3}$ deoxythymidylate incorporated): O, T4v₁; ●, T4v₁x.

FIG. 6. Effect of MC on the DNA polymerase reaction. The reaction mixture (0.3 ml) was composed of the following: 67 mM Tris-chloride (pH 8.6), 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 16.6 mM ammonium sulfate, 6.7 μM EDTA, 0.33 mM NaF, 167 μg of bovine plasma albumin per ml, 0.24 mM heat-denatured salmon sperm DNA, 0.033 mM each of dCTP, dATP, dGTP, and 3H -dTTP (10⁶ counts per min per μmol), 2.6 units of T4D-, or 3.1 units of T4x-DNA polymerase (DEAE-cellulose fraction), and varying concentrations of MC. The mixtures were incubated at 37 C for 30 min. Symbols: O, T4D; ●, T4x.

TABLE 1. DNA polymerase reaction with DNA templates treated by MMS^a

Time of treatment of DNA with MMS (min)	DNA polymerase act.	
	T4x ⁺ (%)	T4x (%)
0	100	100
5	105	96
12	104	90
20	102	86
40	95	84
60	88	83

^a Modification of DNA templates was performed as described in the text. Conditions of the reaction were essentially identical with the legend to Fig. 6 except that MC was omitted. All points are averages of three to five independent experiments and activity of the control was represented as 100.

used as template. However, in this case too, no difference is observed between x^+ and x enzymes.

In addition, we compared other properties of DNA polymerase and found that x^+ and x enzymes exhibit the same heat inactivation curves and the optimum ionic strength for the reaction (Table 2). Assay of polymerase-associated 3' → 5' exonuclease activity with 3'-terminally labeled salmon sperm DNA re-

vealed that the preparation of x^+ -infected cells has 0.75 unit of exonuclease activity, whereas that of x -infected cells has 0.51 unit of activity. Thus, there is no significant difference between DNA polymerases from x^+ - and x -infected cells.

Effect of UV on x^+ and x DNA synthesis.

Cells were infected with UV-irradiated T4D or T4x and DNA synthesis during the first 30 min of infection was determined. Figure 7 shows that DNA synthesis of T4x is more sensitive to UV than is that of T4x⁺ by a factor of 2.

When DNA irradiated with UV (3×10^3 to 1.8×10^4 ergs/mm²) was used as template for the DNA polymerase reaction, the reaction is considerably inhibited (Fig. 8). However, no difference is observed between the x^+ and x enzymes. The extent of the inhibition observed here is less than that observed in infected cells, although the DNA template was irradiated with a higher UV dose than used for irradiation of the phage.

TABLE 2. Enzymatic properties of T4x⁺- and T4x-DNA polymerase

Conditions	DNA polymerase act			
	T4x ⁺		T4x	
	Counts per min	%	Counts per min	%
NH ₄ Cl concn ^a				
0 M	1,772	69	1,869	54
0.05	2,574	100	3,413	100
0.10	1,712	67	2,789	82
0.15	627	24	1,109	33
0.20	165	6	256	8
Time of preincubation at 42 C (min) ^b				
0	2,510	100	3,235	100
30	678	27	802	25
60	212	9	257	8

^a Effect of salt concentration on DNA polymerase activity. Reactions were conducted under the standard assay conditions as described in the legend to Fig. 6, except for the omission of (NH₄)₂SO₄. NH₄Cl was added at the concentrations indicated. The highest activity, with 0.05 M NH₄Cl, represented 3.4 nmol and 2.6 nmol of acid-insoluble nucleotide in 30 min with T4x- and T4x⁺-enzyme, respectively, and each was taken as 100%.

^b Heat inactivation of DNA polymerase. About 2 units of enzyme were incubated at 42 C in 0.05 M Tris-chloride (pH 7.5), 0.1 M (NH₄)₂SO₄, and 2 mg of bovine plasma albumin per ml. At the times indicated, samples were chilled and 2-mercaptoethanol was added to give 0.01 M. Assay for polymerase was conducted at 37 C for 10 min in the standard assay mixture.

FIG. 7. DNA synthesis in the cells infected with UV-irradiated phage. *E. coli* P3478 cells were grown to 6×10^8 cells/ml in phosphate-minimized Tris medium (pH 7.5) at 37 C and infected with T4D or T4x, which had been irradiated with varying doses of UV. After 2 min of infection, ^{32}P was added to give a concentration of $1 \mu\text{Ci/ml}$ and aerated for 30 min at 37 C. Incorporations of ^{32}P into DNA were determined as stated in the legend to Fig. 1. Symbols: O, T4D; ●, T4x.

DISCUSSION

MC is an antibiotic whose mode of action has been studied extensively. It has been shown that MC inhibits DNA synthesis without appreciably affecting RNA and protein synthesis in various organisms (22, 24), and that it causes cross-linking of the complementary strands of DNA (15). One of the most remarkable features of MC action is that it distinguishes cellular and viral DNA synthesis; DNA synthesis by virus-infected cells is not inhibited by MC at concentrations sufficient to inhibit DNA synthesis by uninfected cells (18, 22).

Increased resistance to MC of DNA synthesis in virus-infected cells may be explained by one or a combination of the following mechanisms. (i) Viral DNA is less damaged by MC than is host DNA, since the former has a smaller target size compared with the latter. (ii) Virus-infected cells have a permeability barrier which prevents access of MC to its sites of action. (iii) Infection with virus induces a mechanism to repair MC-induced damages or a new DNA-synthesizing system that is resistant to MC or MC-induced damages.

It was shown, in the present experiments, that the sensitivity to MC of viral DNA synthe-

sis is controlled, at least in part, by the viral genome; T4x DNA synthesis is more sensitive to MC than is that of the wild type. This phenomenon cannot be explained by the target size, since x^+ and x phages contain almost the same functional units of DNA. Although it is difficult to eliminate the possibility that the x mutation affects cell permeability, other properties of x (e.g., increased radiation sensitivity) cannot be explained by the second mechanism.

Data presented in this paper are compatible with the idea that the DNA-synthesizing system of x is more vulnerable to MC or MC-induced damages than is that of x^+ . Since x exhibits increased sensitivity to UV, ionizing radiation, and alkylating agents as well, the hypothesis may be stated as follows. Some damages in DNA, which can be bypassed by the DNA replicating machinery of x^+ -infected cells, block the DNA synthesis of x -infected cells.

Thus, attempts have been made to compare the DNA polymerases from x^+ - and x -infected cells. There is evidence that T4-induced DNA polymerase is involved in replication and possibly repair and recombination of phage DNA (1,

FIG. 8. DNA polymerase reaction with UV-irradiated DNA templates. Heat-denatured, UV-irradiated salmon sperm DNA was prepared as described in Materials and Methods and subjected to the T4 DNA polymerase reaction as templates. Experimental conditions were essentially identical with the legend to Fig. 6 except that MC was omitted. Curves indicate the relative priming activity as a function of UV-dose, with which DNA templates had been irradiated. Symbols: O, T4x⁺ polymerase; ●, T4x polymerase.

21). Moreover, it was shown that α mutation maps close to gene 43 (29), the gene coding for DNA polymerase (6). Thus the possibility arises that the α mutation is inside the polymerase gene and alters properties of DNA polymerase itself.

As far as the present experiments go, however, there is no indication that T4 α DNA polymerase is abnormal. DNA polymerase from T4 α -infected cells is as resistant to MC as that of wild type, and the two enzymes exhibit the same heat inactivation curves and optimum ionic strength for the polymerization reaction. It was shown, moreover, that reactions by α^+ and α enzymes are equally affected by modification of the DNA template with MMS or UV. The extent of inhibition of the DNA polymerase reaction by UV is almost comparable to the inhibition observed with the calf thymus DNA polymerase system previously reported by Bollum and Setlow (3), who suggested that such blocking is caused by pyrimidine dimers. More definite evidence to indicate that the lesions blocking the polymerization process are pyrimidine dimers and that polymerization can proceed past the UV lesions except dimers was obtained with ϕ X174 DNA and *E. coli* DNA polymerase by Poddar and Sinsheimer (20).

What has gone wrong with the α mutation? An alteration of DNA polymerase, if any, might be too subtle to find out by the procedures used in these experiments. Or, an alteration may reside in another enzyme or factor which is also involved in replication of DNA. Therefore, it seems important to investigate the problem by using membrane-containing systems which more nearly approximate the properties of *in vivo* replication.

Finally, it should be noted that the sensitivities to MC of T4 wild type and the ν mutant are identical, namely that DNA synthesis by T4 ν -infected cells is as resistant to MC as that of T4D-infected cells, and also ν^+ and ν phages produced in the presence of MC exhibit almost the same infectivity. This implies that the ν gene product, T4 endonuclease V, does not function to repair MC-damaged DNA. It has been shown that *E. coli* *uvr* genes, whose function can be replaced by the ν gene product (14, 27), are involved in repair of MC-induced damage (19). It seems of interest to see whether the apparent difference is due to different specificities of T4 and *E. coli* repair enzymes.

ADDENDUM IN PROOF

After this paper was submitted for publication, we were informed that the α mutation resides in the approximate vicinity of genes 41 to 56, but not of

gene 43 (J. W. Drake, Genetics, in press). This is consistent with the present finding that T4 α DNA polymerase is not abnormal.

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