Isolation of an *E. coli* Strain with a Mutation affecting DNA Polymerase

by

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By testing indiscriminately several thousand colonies of mutagenized *E. coli*, a mutant has been isolated that on extraction proves to have less than 1 per cent of the normal level of DNA polymerase. The mutant multiplies normally but has acquired an increased sensitivity to ultraviolet light.

KORNBERG's discovery of an enzyme that could faithfully copy DNA *in vitro*[1] was a crucial step in the history of molecular biology because it firmly established the fact that only a small part of a cell's DNA is needed to code for a mechanism that can duplicate the whole. Whether this is the enzyme responsible for DNA duplication *in vivo* was rightly thought, at that time, to be of secondary importance. Since then, however, circumstantial evidence has accumulated suggesting that, at least in bacteria, this particular enzyme is used for the repair of DNA rather than for its duplication. The various mutants of *Escherichia coli* and *Bacillus subtilis* that are unable to duplicate their DNA at high temperature have all been shown to contain normal polymerase and normal deoxyribonucleoside triphosphate pools at the non-permissive temperature[2–6], and at least one of them has been shown to carry out repair synthesis at high temperature[7]. Repair replication and the process of DNA duplication apparently differ in the extent to which they discriminate against 5-bromouracil as an acceptable substitute for thymine, suggesting that the two reactions involve different polymerases[8]. Finally, the 5′-exonucleolytic activity, recently shown to be an intrinsic property of the *E. coli* polymerase[9], is clearly a desirable attribute for an enzyme responsible for excision and repair but is of no obvious advantage for an enzyme carrying out semiconservative replication.

These and other less persuasive arguments prompted us to look for mutants of the polymerase, in the hope that they would either establish a role for the polymerase in DNA duplication or exclude it and, at the same time, provide convenient strains in which to search for the right enzyme. Although we have not succeeded in these more distant objectives, we have isolated such a mutant and here describe the method of isolation and some of its properties. The accompanying article describes a genetic study of the mutation.

The Selective Procedure

The successful isolation of mutants of *E. coli* lacking ribonuclease I[10] demonstrated that it is possible to find the mutant one wants simply by testing individually several hundred colonies grown from a heavily mutagenized stock. Because we wished to avoid having to guess what symptoms, if any, would result from a lack of DNA polymerase, we decided to follow that example and assay the polymerase in clones of a mutagenized stock until we found what we were looking for. We had to allow for the possibility that the mutation we sought might be a conditional lethal, so we began by assaying at 45° extracts made from clones grown at 25° or 30°; later we tested clones grown at 37°, thinking that temperature-sensitive mutants of the polymerase might be more readily detectable if the enzyme had been assembled at a higher temperature. As it turned out, the mutant we eventually isolated would have been found whatever approach had been adopted, and we shall therefore simply give the history of the mutant when we describe its isolation and properties.

Extraction of Polymerase

Because we expected to have to test many hundred colonies, we required a very simple method for preparing extracts. In addition, we needed a procedure which made the bacteria incapable of incorporating deoxyribonucleosides, to ensure that labelled triphosphates could not enter DNA by way of breakdown to nucleosides and incorporation by those few cells that might have survived the extraction procedure. These two requirements were satisfied by the slight modification of a method devised for extracting polysomes, using the non-ionic detergent Brij-58 (ref. 11). *E. coli* is suspended at a concentration of about 3×10^9/ml. in ice cold 10 per cent sucrose 0·1 M Tris (*p*H 8·5); lysozyme and EDTA are added to final concentrations of 50 μg/ml. and 0·005 M, respectively, and the mixture is kept on ice for 30 min; addition of a mixture of Brij and $MgSO_4$ (at room temperature) to give final concentrations of 5 per cent and 0·05 M, respectively, results in partial clearing; following centrifugation (1,500*g* for 30 min), the deposit contains 99·9 per cent of the DNA and the supernatant contains the polymerase, which may then be assayed simply by adding sonicated calf thymus DNA (to 50 μg/ml.) and the four deoxyribonucleoside triphosphates (to a final concentration of 4 nmoles/ml. dATP, dGTP, dCTP and 2 nmoles/ml. ^{3}H-TTP).

This extraction procedure demonstrates one point of interest: any method of lysis that liberates fragmented DNA will automatically create sites for the attachment of polymerase and therefore cannot give a true picture of the location of the polymerase *in vivo*[12]. Extraction with Brij yields cells which still contain their DNA but, on resuspension, have little if any ability to incorporate deoxyribonucleoside triphosphates. Because Brij apparently does not dissociate polymerase from its template (the polymerase being assayable in the presence of Brij), we can conclude that most of the polymerase in *E. coli* is normally not attached to DNA but lies free within the cell—as might befit an enzyme awaiting the summons to repair synthesis. This conclusion is supported by the observation that when *E. coli* segregates daughter cells which lack DNA these cells nevertheless retain their full quota of DNA polymerase[13,14].

Isolation of the Mutant

E. coli W3110 *thy*⁻, growing in minimal medium, was washed and suspended in 0·15 M acetate (*p*H 5·5), treated with N-methyl-N′-nitro-N-nitrosoguanidine (1 mg/ml.) for 30 min, and then centrifuged and suspended in Penassay broth[15]. Following growth at 25° C for 18 h, the culture was plated; after incubation overnight at 37° C, the colonies were picked into 1 ml. lots of Penassay broth which were incubated overnight at 37° C and then centrifuged and extracted with lysozyme and Brij.

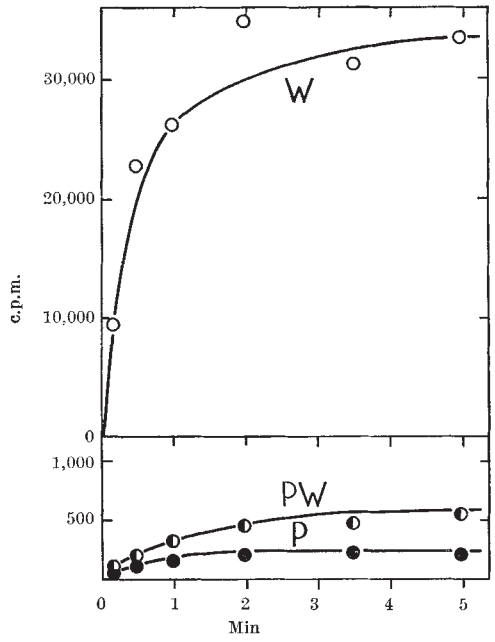

Fig. 1. Triphosphate incorporation by extracts of the parent strain (W), the mutant strain (P) and a mixture of 99 per cent mutant and 1 per cent parent (PW). *E. coli* W3110 *thy*⁻ and the mutant derivative, p3478, were grown with aeration in Penassay broth at 37° to about 5×10^8/ml. Each culture was then chilled, centrifuged and suspended in 0·1 M Tris–0·01 M $MgSO_4$ (*p*H 7·4) at a concentration of 6×10^9/ml. A mixture of 1 per cent parent strain, 99 per cent mutant strain was prepared. This and the two unmixed suspensions were centrifuged and suspended in Tris-Mg^{2+} at a concentration of 1×10^{10}/ml. The three suspensions were disrupted by sonic vibration and mildly centrifuged (1,000*g* for 10 min). To 0·9 ml. of each supernatant at 25° 0·1 ml. sonicated calf thymus DNA was added (final concentration 20 μg/ml.) and, 5 min later, 0·3 ml. triphosphate solution (final concentrations 100 nmoles/ml. dGTP, dATP, dCTP, and 0·6 nmoles/ml., 2·5 μCi/ml., ^{3}H-TTP). Samples of 0·2 ml. were taken from each reaction mixture into 5 ml. 5 per cent trichloroacetic acid–1 per cent sodium pyrophosphate[16]. These samples were then washed on Whatman GFA filters with 5 per cent trichloroacetic acid and with 5 per cent acetic acid, dried and counted in a scintillation counter.

After testing a few thousand colonies in this way we found a clone, p3478, that appeared to lack polymerase activity. It was therefore tested again using a more conventional method for extracting the enzyme. According to this test (Fig. 1), extracts of the mutant have 0·5–1·0 per cent of the normal activity. This decrease in activity does not seem to arise from the presence of an inhibitor.

Some Properties of the Mutant

As far as we can determine, the mutant multiplies at the same rate as the parent strain, in minimal and complete media, and at temperatures from 25° to 42° C. On plating, it forms slightly smaller colonies than those of the parent strain, and occasionally it seems to have difficulty in getting out of stationary phase, but we have not investigated further either of these phenomena.

Parent and mutant are equally susceptible to infection with T4, T5, T7 and λ bacteriophages. When converted to spheroplasts, they are equally susceptible to φX174 DNA and produce equal yields of phage (personal communication from David Dressler). This finding was somewhat surprising, but it should be remembered that all stages in the replication of φX174 DNA are temperature sensitive in a mutant that is temperature sensitive for normal DNA replication[17] but not for repair synthesis[7].

With regard to host cell reactivation, there is no detectable increase in the rate of inactivation of T7 by ultraviolet light, when the survivors are assayed on the mutant rather than the parent. Thus the mutant is *hcr*⁺.

The mutant has a marked increase in sensitivity to ultraviolet light. For convenience, this effect will be documented in the following article[18], where the sensitivities of various derivative strains are compared.

The parent strain will form colonies normally in the presence of 0·04 per cent methylmethanesulphonate, whereas the mutant plates with an efficiency of about 10^{-7}. We assume that these rare methylmethanesulphonate-resistant cells are revertants that have either arisen spontaneously or been created by the methylmethanesulphonate. Because every one of twenty such independently arising revertants exhibited normal sensitivity to ultraviolet light and had normal or near-normal levels of polymerase, it is clear that the three basic properties of the mutant (UV^s, MMS^s and lack of polymerase) are the result of a single mutational step.

Repair or Replication

The accompanying article[18] demonstrates that we are dealing with an amber mutation which is recessive in partial diploids. We assume that it is in the gene coding for DNA polymerase, although proof will require the demonstration that it—or other similar mutations—results in changes in the polymerase protein. Because the mutation produces an increased sensitivity to ultraviolet light, it seems likely that recovery from the effects of ultraviolet light is partly the responsibility of this polymerase.

Unfortunately, it is not going to be easy, by a study of this or other such mutants, to show that this polymerase plays no part in normal DNA duplication. Because *E. coli* contains several hundred polymerase molecules per bacterium[19], the residual activity found in extracts of our mutant could represent perhaps 5–10 molecules per cell—a number that could well be sufficient for normal duplication. Even if we could somehow prove that the residual activity were entirely that of another enzyme (in other words, that this amber mutation is not measurably leaky), we should still not have proved that duplication is carried out by some other enzyme, for it could readily be argued that those few polymerase molecules concerned with duplication are necessarily incorporated into some larger enzyme complex the activity of which is not assayable *in vitro*. It could even be argued that more of the polymerase molecule must be intact for it to serve as a repair enzyme (and, incidentally, to survive extraction) than for it to act when part of the replicating machinery. We therefore believe that the question will be resolved either by engineering a total deletion of the polymerase gene or by determining, in some direct manner, which enzymes and what precursors are used for normal DNA duplication. It is our hope that each of these exercises will have been made easier now that the polymerase gene has probably been located[18] and a mutant is generally available.

We thank Dr Raymond Gesteland (who pioneered this kind of mutant hunt) for encouragement; Dr David Dressler for testing our mutant with φX174 and for permission to cite his results; and Drs Julian and Marilyn Gross for arranging to stay on at Cold Spring Harbor to conduct most of the experiments reported in the next article.

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Genetic Analysis of an *E. coli* Strain with a Mutation affecting DNA Polymerase

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The mutation affecting DNA polymerase activity in the mutant strain isolated by De Lucia and Cairns is located between *metE* and *rha*, at approximately 75 minutes on the *Escherichia coli* chromosome. The mutation is recessive to the wild-type gene in partial diploids. It is an amber nonsense mutation which responds to the suppressors Sul$^+$, Sull$^+$ and Sulll$^+$. Strains carrying the mutation are not deficient in carrying out genetic recombination.

De Lucia and Cairns[1] have reported the isolation of a mutant strain of *Escherichia coli* which differs from the parent strain W3110 *thy*$^-$ in three characteristics: greatly reduced DNA polymerase activity in extracts, increased sensitivity to ultraviolet irradiation (UV) (see Fig. 2, this article) and increased sensitivity to methylmethanesulphonate (MMS). Evidence presented by De Lucia and Cairns[1], and here, indicates that these properties are the result of a single lesion probably located in the structural gene for DNA polymerase. We propose that the corresponding gene locus be termed *polA*, and the particular mutation studied here, *polA*1.

In order to determine the approximate position of *polA* on the *E. coli* chromosome, we made donor derivatives of parent and mutant strains by introducing into them an *Flac* factor. The *Flac* factor used carries a dominant mutation in the *lac* repressor gene which renders cells carrying it non-responsive to inducer (i^s)[2], so that derivatives harbouring the factor could be detected by their inability to ferment lactose. The presence of the *Flac* factor did not alter the radiation sensitivity or polymerase activity of the mutant. The *Flac* factor was found to mobilize the chromosome with equal efficiency in the parent and mutant strains. The origin and direction of chromosome transfer resulting from mobilization by *Flac*[3] are shown in Fig. 1.

In a cross between the recipient strain JC411 *leu*$^-$ *metB*$^-$ *argG*$^-$ *his*$^-$ *gal*$^-$ *str*^{r} and the *Flac* derivative of the mutant strain, recombinants inheriting *leu*$^+$, *metB*$^+$, *argG*$^+$, *his*$^+$ or *gal*$^+$ from the donor, and *str*^{r} from the recipient (see Fig. 1) were selected and scored for concomitant inheritance of UV sensitivity. Sixty per cent of the *metB*$^+$ *str*^{r} recombinants were UV sensitive. None of the recombinants of the other classes tested were UV sensitive. This result indicates that *polA* is located in the *metB* region of the chromosome.

*polA*1 is an Amber Mutation

Extracts of fourteen *metB*$^+$ *str*^{r} recombinants that had also inherited UV sensitivity, and six that had not, were tested for DNA polymerase activity. All the UV sensitive recombinants were found to have reduced polymerase activity whereas the UV resistant recombinants had normal levels. Extracts of the UV sensitive recombinants, however, contained about 8 per cent of wild type activity compared with levels of 0·5 per cent to 1 per cent detected in the original *polA*1 mutant. They were also found to have a degree of UV sensitivity intermediate between wild type strains and the original *polA*1 mutant strain. We therefore suspected that the recipient strain JC411 might carry a suppressor mutation that partially restores the activity of *polA*1. We tested the growth of a set of amber and ochre mutants of phage T4 on JC411 and W3110. JC411 was found to permit the growth of certain of the amber mutants but not of the ochre mutants and so presumably contains an amber suppressor. W3110 did not produce any detectable suppression.

In order to test the inference that *polA*1 is an amber mutation we introduced a *lacZ*$^-$ amber mutation into the original *polA*1 strain. This enabled us in subsequent

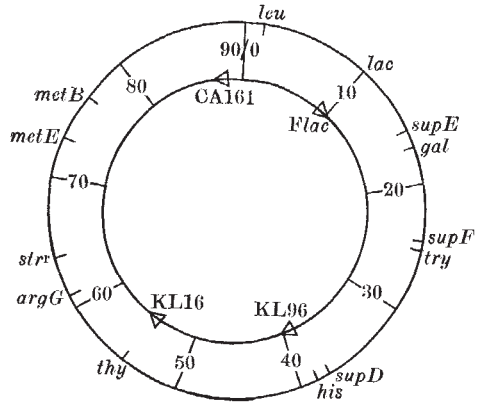

Fig. 1. Linkage map of *E. coli* showing the position of certain loci used in the present study (outer circle) and the origin and direction of transfer of three Hfr strains and of mobilization by the *Flac* factor (inner circle). The chromosome is divided into nine segments corresponding to intervals of 10 minutes on the standard map[4]. Symbols for genetic markers are those listed by Taylor and Trotter[4].