DNA Synthesis in Gamma-irradiated Recombination Deficient [Strains of *Escherichia coli*

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SUMMARY

DNA synthesis in *recA* and *recA exrA* strains of *Escherichia coli* is strongly inhibited without delay by low doses of gamma-radiation. The synthesis that does occur in these strains appears to take place mainly on chromosomes that have not received a lethal hit. Low doses produce much less inhibition of DNA synthesis in *exrA*, *recB*, *recA recB* and wild-type strains. We conclude that the *recA*⁺ gene product acts immediately, certainly before DNA replication, in the repair of lesions produced by gamma-irradiation. In *recB* bacteria the *recA*⁺ gene product is not required for continued DNA synthesis, although the synthesis which occurs in *recA recB* strains may be abnormal since it is not reflected in increased survival. The *exrA*⁺ gene product is not necessary for continued DNA replication, suggesting that its time of action in repair may be at a later stage.

INTRODUCTION

The most easily observed effect of gamma-irradiation on bacterial DNA is the introduction of single strand breaks. One class of breaks is transient, repair occurring rapidly by a process requiring Kornberg polymerase (Town, Smith & Kaplan, 1971) whereas another rarer class undergoes repair slowly by a process requiring the *recA*⁺, *recB*⁺ and *exrA*⁺ gene products (McGrath & Williams, 1966; Morimyo, Horii & Suzuki, 1968; Kapp & Smith, 1970). Although it is not known whether this slow repair process occurs before, during or after DNA replication, its slowness suggests that gaps could persist until replication. Moreover, the effects of gamma-irradiation on conjugation (Green, Bridges & Riazuddin, 1971) suggest that *recA*⁺-dependent repair is completed during or after replication of DNA bearing the lesion. Cairns & Davern (1966), however, have found that one ³²P-induced double strand break anywhere in the chromosome is sufficient to inhibit DNA synthesis immediately in a presumed *recA* strain and this result would imply that *recA*⁺-dependent repair of ³²P damage must occur before DNA replication can continue.

In an attempt to clarify the situation we have examined DNA synthesis immediately after gamma-irradiation in *recA*, *recB*, *recA recB*, *exrA* and *recA exrA* strains of *Escherichia coli*. Our results clearly separate the *recA*⁺, *recB*⁺ and *exrA*⁺ functions.

METHODS

Bacterial strains. *Escherichia coli* K-12 AB1157 (u.v.-resistant) and its derivatives AB2463 *recA13*, AB2470 *recB21* (Howard-Flanders & Boyce, 1966), JC5495 *recA13 recB21* (Willetts & Clark, 1969), PAM5717 *exrA* (Donch, Green & Greenberg, 1968) and CM293 *recA56 exrA* were used.

The strain CM293 *recA* *exrA* was a *his*⁺ recombinant selected from a mating between strain JC5088 Hfr *recA56* and strain PAM5717 *exrA*. In this cross *exrA*⁺ is transferred as an extreme distal marker and mating was interrupted at 20 min before the *exrA*⁺ gene could be transferred. The recombinant was classified as *recA* on the basis of its greater u.v. sensitivity and altered DNA synthesis after gamma-irradiation. It was presumed *exrA* because mating had been interrupted, because it retained the auxotrophic markers of PAM5717, and because it retained the robust growth of PAM5717 rather than the slower growth of a *recA* strain.

We are indebted to Dr A. J. Clark and Dr P. Howard-Flanders for the other strains used.

Cultural conditions. Cultures were grown at 37 °C with shaking in M9 minimal medium supplemented with 0.4% (w/v) glucose and 1% (w/v) Difco Casamino acids. Overnight stationary cultures were diluted into fresh medium (1/100 AB1157, PAM5717, CM293; 1/50 AB2463; 1/25 AB2470, JC5495) and grown for 3 h.

Irradiation. Samples of 3 h cultures were irradiated at room temperature with aeration in a ⁶⁰Co 'hot-spot' source at a dose rate of 4.5 krad/min. Viability of irradiated samples was determined on plates of Oxoid Nutrient Broth no. 2 solidified with 1.5% Davis New Zealand Agar.

Radiochemicals. [³H-methyl]thymidine (specific radioactivity 26 Ci/mM), [2-¹⁴C]uracil (60 mCi/mM), [8-¹⁴C]adenine (60 mCi/mM), [8-¹⁴C]guanine (55 mCi/mM), [2-¹⁴C]thymidine (60 mCi/mM), and [³H-methyl]thymidylic acid (12 Ci/mM) were obtained from The Radiochemical Centre, Amersham, Buckinghamshire.

Measurement of DNA synthesis

(a) *Incorporation of [³H]thymidine.* Immediately after irradiation, 3 ml of each sample was added to 7 ml of warmed fresh medium containing 10 µCi [³H-methyl]thymidine. At 1, 2, 3, 4, 5, 6, 8 and 10 min, 1 ml samples were pipetted into 4 ml ice-cold 10% (w/v) trichloroacetic acid. The acid insoluble fraction was collected and assayed for radioactivity as described previously (Green, Gray, Murden & Bridges, 1971).

(b) *Incorporation of other precursors.* Measurements of the rate of uptake of radioactivity into DNA from labelled adenine, guanine and uracil were performed in the presence of 100 µg/ml rifampicin, in order to prevent incorporation into RNA (Wehrli, Knusel, Schmid & Staehelin, 1968). The antibiotic was added as a concentrated methanolic solution 5 min before irradiation. After irradiation the cultures were rewarmed to 37 °C and the samples were added to the labelled precursor to give a final concentration of 0.25 µCi/ml. Samples of 1 ml were removed at intervals and added to 2 ml 1 N-KOH and incubated at 37 °C for 18 h in order to hydrolyse any residual labelled RNA (Gray & Midgley, 1971). The labelled DNA was precipitated with an excess of ice-cold 10% (w/v) trichloroacetic acid and assayed for radioactivity as before.

Rifampicin was a kind gift from Lepetit Pharmaceuticals Ltd, Maidenhead, Berkshire.

Measurement of DNA breakdown. Breakdown of DNA was estimated by observing the loss of trichloroacetic acid precipitable radioactivity from labelled cells. Cultures were labelled for 10 min with 1 µCi/ml [³H-methyl]thymidine, filtered on Millipore filters (0.45 µ pore size), washed on the filter with an equal volume of distilled water, resuspended in fresh medium and incubated for 10 min to allow self-chasing to occur. Almost identical results were obtained when cultures were randomly labelled with [³H-methyl]thymidylic acid (Breitman, Bradford & Cannon, 1967).

Fig. 1. Effect of various doses of aerobic γ -irradiation (0 to 5 krad) on the uptake of $[^3\text{H}]$ thymidine into the cold acid insoluble fraction of AB2463.

RESULTS

The rate of DNA synthesis immediately after irradiation was estimated by observing the rate of incorporation of tritiated thymidine into the cold acid insoluble fraction. Over the 10 min period, uptake was found to be linear in all cases (Fig. 1). Values for the rate of uptake of thymidine were obtained by applying linear regression analysis to the results. Rates of uptake, expressed as a percentage of that in an unirradiated sample, are presented in subsequent figures as a function of radiation dose.

Compared with the wild-type, DNA synthesis in the *recA* strain was extremely sensitive to gamma radiation, the kinetics suggesting immediate inhibition by a single hit (strand break?) with a target size about the same as that for lethality (Fig. 2a, b). If the same lesion both kills and immediately stops DNA synthesis in the *recA* strain, the residual synthesis observed should be almost entirely on chromosomes escaping such a lesion. An experiment supporting this conclusion is described below.

The *recB* strain did not show the high sensitivity of the *recA* strain and at higher doses DNA synthesis was somewhat more resistant than in the wild-type. It is significant that the high sensitivity of the *recA* strain to immediate inhibition requires the *recB*⁺ gene product since DNA synthesis in the *recA recB* strain was as resistant as that in the *recB* strain.

In contrast to the *recA* strain, immediate post-irradiation DNA synthesis in the *exrA* strain was almost as resistant to radiation as in the wild-type strain (Fig. 3a). The *recA exrA* double mutant, unlike the *recA recB*, showed a high sensitivity similar to that of the *recA*

Fig. 2. (a) Effect of aerobic γ -irradiation on the rate of uptake of [³H]thymidine into cold acid insoluble material of strains AB1157 wild-type, AB2463 *recA*, AB2470 *recB* and JC5495 *recA recB*; the rate is expressed as a percentage of the rate of uptake into an unirradiated control culture. (b) Effect of aerobic γ -irradiation on survival of the same strains.

Fig. 3. (a) Effect of aerobic γ -irradiation on the rate of uptake of [³H]thymidine into cold acid insoluble material of strains AB1157 wild-type, PAM5717 *exrA* and CM293 *recA exrA*; the rate is expressed as a percentage of the rate of uptake into an unirradiated control culture. (b) Effect of aerobic γ -irradiation on survival of the same strains.

Table 1. *Rate of DNA synthesis after gamma-irradiation as measured by uptake of different bases*

Strain AB2463 *recA* received 0 or 2 krad gamma-irradiation and was incubated with the different bases for 40 min as described in Methods.

The results for [^3H]thymidine incorporation over a 10 min period, which were used to calculate Fig. 2*a*, are included for comparison.

	Rate of DNA synthesis after 2 krad relative to unirradiated control (%)				Mean
	Experiment no.				
	1	2	3	4	
[³ H]Thymidine over 10 min	34.9	30.8	35.7	37.0	34.6
[¹⁴ C]Thymidine over 40 min	28.4	29.3	21.0	—	26.2
[¹⁴ C]Adenine	32.1	28.9	24.0	—	28.3
[¹⁴ C]Guanine	32.8	35.0	33.5	—	33.7
[¹⁴ C]Uracil	38.0	35.0	34.2	—	35.7
([¹⁴ C]Uridine was used in one experiment)					

strain. Despite the different sensitivities of immediate post-irradiation DNA synthesis, all the *Rec* and *Exr* strains were about four times more sensitive than the wild-type to the lethal action of gamma radiation (Fig. 2*b*, 3*b*). Thus, in these strains post-irradiation DNA synthesis is not correlated with survival in any simple way.

A number of other strains bearing comparable mutations has also been tested and found to behave similarly to those for which detailed data have been given; they include NG30 *recA* and JC5088 *recA* (which resembled AB2463 *recA*), JC5489 *recC* (which resembled AB2470 *recB*), and AB2494 *lex* (which resembled PAM5717 *exrA*). Our results are therefore likely to be attributable to the mutations studied rather than to any unsuspected strain differences. We noticed, however, that DNA synthesis in these strains after gamma-irradiation was almost the reciprocal of DNA breakdown (W. J. H. Gray, M. H. L. Green & B. A. Bridges, unpublished observations). It was thus possible that the apparent inhibition of DNA synthesis which we observed might have been due to incorporation of (cold) breakdown products in preference to exogenous [^3H]thymidine. We therefore examined the uptake of ^{14}C -labelled uracil, adenine, guanine and thymidine into DNA, as described in Methods. Uracil is incorporated into DNA as both thymine and cytosine, and for both of these bases the competition with breakdown products should be different from that for exogenous thymidine. A similar argument also applies to adenine and guanine, since their pool sizes differ (Buchwald & Britten, 1963). Nevertheless we obtained results for incorporation of these bases into strain AB2463 *recA* after gamma-irradiation which were substantially similar to those observed for [^3H]thymidine incorporation (Table 1).

Moreover, Green, Bridges & Riazuddin (1971) have shown that under conditions where normal DNA synthesis is totally inhibited by gamma-irradiation in *recA* male and female strains, conjugation DNA synthesis may still be detected. This implies that if DNA synthesis were occurring in gamma-irradiated *recA* strains it would be detected.

Further evidence suggesting that the inhibition of DNA synthesis which we observed was genuine came from a comparison of the stability of DNA synthesized after irradiation with that of existing DNA. It can be seen from Fig. 4 that in a *recA* culture the stability of DNA synthesized after irradiation (2 krad) corresponds more closely to the stability of DNA in unirradiated cells, than to the stability of existing DNA in an irradiated culture. Moreover, after a second dose, this post-irradiation DNA breaks down to almost exactly the same extent

Fig. 4. The stability of DNA synthesized after fractionated doses of γ -radiation in strain AB2463 *recA*. Cultures were labelled for 10 min as described in Methods, the label was removed and the cultures allowed to self-chase for 10 min. Cultures were irradiated immediately before or 10 min after labelling. Loss of acid insoluble radioactivity was measured from 10 min after label was removed. ●, Culture receiving no irradiation; ○, culture receiving 2 krad after labelling; ▲, culture receiving 4 krad after labelling; △, culture receiving 2 krad before labelling; ×, culture receiving 2 krad before labelling and a further 2 krad after labelling.

as existing DNA which has received a single dose. Thus, the DNA synthesized after irradiation in a *recA* strain behaves as if it has largely been synthesized on the undamaged fraction of chromosomes in the population.

It is also clear from this experiment that the apparent rate of DNA synthesis is not greatly reduced by recycling of DNA breakdown products. If we consider the fate of the existing DNA, at least 55% is broken down and released into the medium after irradiation (Fig. 4) and about 45% remains stable. Most of this 45% is required to act as template for the stable DNA synthesized after irradiation. Following 2 krad irradiation, 34.6% as much exogenously labelled DNA is synthesized as in the unirradiated culture (Fig. 2a). Of this DNA 79.6% is stable (Fig. 4), which implies that 27.5% ($34.6 \times 79.6/100$) of the existing DNA is needed to act as stable template for the new stable exogenously labelled DNA. Thus 8.7% $-(45 - 27.5)/2$ of the existing DNA is recycled into stable DNA and 8.7% of the existing DNA must act as a stable template for this recycled DNA. From several experiments of this type we calculate that not more than 5 to 10% of the existing DNA is recycled. A similar figure was obtained for the DNA synthesis resistant strain *exrA*.

We may therefore be slightly underestimating the rate of DNA synthesis after irradiation. However, the relative levels observed remain unchanged.

DISCUSSION

The *recA*⁺-dependent repair of single strand DNA breaks produced by ionizing radiation is known to be a slow process requiring the equivalent of approximately one replication time for completion (McGrath & Williams, 1966; Smith, 1971). The high radiation sensitivity of the initial rate of DNA synthesis that we have observed in the *recA* and *recA exrA* strains implies an immediate requirement for the *recA*⁺ gene product if DNA replication is to continue. This requirement appears to be waived in the absence of the *recB*⁺ gene product, although the resulting synthesis may be abnormal as repair of single strand breaks certainly does not occur (Smith, 1971). Since the *recB*⁺ gene product is also necessary for radiation-induced DNA breakdown in *recA* strains (Kapp & Smith, 1970), inhibition of synthesis and breakdown may be causally related one to another.

The time of action of the *recB*⁺ gene product is problematical. Although the *recB*⁺ gene product is involved in immediate inhibition of DNA synthesis after irradiation of a *recA* strain, this could be a pathological condition and does not necessarily imply early action during repair in a *recA*⁺ strain. The *exrA*⁺ gene product appears not to affect post-irradiation DNA synthesis and may be involved in some subsequent stage of repair.

A hypothesis consistent with our results is that repair of ionizing radiation damage occurs in at least two steps widely separated in time; an immediate action of the *recA*⁺ gene product followed some time later by the action of the *exrA*⁺ gene product (quite possibly at or after the time of replication of the damaged region). If continued DNA synthesis is necessary for repair to occur it is conceivable that the *recA* strain is sensitive because it cannot continue to synthesize on a damaged chromosome rather than because the *recA*⁺ gene product has any direct role in the actual repair steps. We can not, of course, discount the possibility that any of these gene products (including the *recA*⁺) have more than one point of action in the repair sequence. Our results are essentially in agreement with those of Cairns and Davern (1966) for ³²P decay, implying an immediate interaction between a lesion and a replication point remote from it. However, their hypothesis that strand breaks might inhibit DNA synthesis by interrupting a driving torque mechanism requires modification. One would have to postulate that only gaps enlarged by uncontrolled *recB* nuclease activity are effective in inhibiting DNA synthesis.

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