Induction by Mitomycin C of recA Protein Synthesis in Bacteria and Spheroplasts*

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The effect of mitomycin C on the synthesis of recA protein in Escherichia coli has been analyzed in a variety of conditions, using an immunoradiometric assay (Paolletti, C., Salles, B., and Giacmoni, P. U. (1982) Biochimie 64, 239-248).

In exponentially growing cultures of E. coli AB 1157, the addition of mitomycin C (5 μg/ml) promotes a 15-fold increase of the content of recA protein with respect to the basal level. Kinetic analysis of this induction shows that the maximum is reached 60 to 90 min after the addition of the drug and then the level decreases. In an uvrA mutant treated with mitomycin C the level of recA protein reaches a maximum within an hour and afterwards it does not decrease.

Treatment of exponentially growing cells with EDTA and lysozyme induces a 3-fold increase of recA protein content, in comparison to the basal level. When such spheroplasts are added with mitomycin or nalidixic acid, a striking increase of the recA protein content in the spheroplast suspension is observed, which tends to level off an hour and a half after the addition of the drugs.

The maximum level of recA protein content is five times the level measured after lysozyme treatment, i.e. 15 times the basal level in exponentially growing cells.

The inducible DNA repair functions, at least those induced by ultraviolet irradiation, can be analyzed by bacteriological techniques since they consist of mutagenesis, induction of prophage in lysogenic cells, and stimulation of reactivation and mutagenesis of DNA-damaged phage.

On the other hand the quantitative analysis of the induction of recA protein by several agents has long been far from being exhausted because of the lack of adequate methods. Up to now the only accessible techniques were either to assay bacterial extracts for proteolytic cleavage of λ repressor (4) and this method only allowed the detection of "activated" recA protein, or to subject these extracts to electrophoresis on gels containing antibodies, and this method is only semi-quantitative. From the data obtained in these ways one could draw some conclusions about the induction of inducible DNA repair functions and about the presence of activated recA proteins, because large amounts of the product of recA gene are synthesized following ultraviolet irradiation (5) and because activated recA protein cleaves λ repressor in vitro (6, 7).

Because of the great importance which is ascribed to the role played by the product of recA gene, it was necessary to develop a simple assay for the quantitative analysis of the content of recA protein, able to detect both activated and inactivated recA protein.

An immunoradiometric assay for the determination of the amount of recA protein in crude bacterial extracts has been recently set up in our laboratory (8). This assay has been designed also in order to determine the dose-response effect of wild type and uvrA E. coli to mitomycin C and to analyze the kinetics of the induction of recA protein in mitomycin C-treated bacteria and spheroplasts. The goal of such experiments is to analyze in a quantitative way the expression of one of the genes controlled by the lexA-recA system.

This paper describes some details of the immunoradiometric assay and reports the kinetic data concerning the induction of recA protein in several E. coli strains. The results are discussed in the light of current models concerning the expression of inducible DNA repair functions.

MATERIALS AND METHODS

Chemicals and Buffers—Nalidixic acid was from Boehringer Mannheim; mitomycin C was from Laboratories Chasen, Paris. Freund incomplete Bacto adjuvant was from Difco Laboratories, Detroit; 125I-Na was from CEA, Saclay. L broth contained in 1 liter, 10 g of Bactotryptone (Difco), 5 g of Bacto yeast extract (Difco), 5 g of NaCl and was titrated to pH 7.2. IRMA-A buffer was 10 mM sodium barbital, 0.5 M NaCl, 5 mM NaN3, 0 μg/ml of bovine serum albumin, 0.1% (v/v) calf serum, titrated to pH 7.3. IRMA-B buffer was the same as IRMA-A without bovine serum albumin and without calf serum. Salmon sperm DNA was purchased from Sigma and it was further purified with three phenol extractions. It was kept in 40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 1 mM Na2EDTA. ATP was purchased from Boehringer Mannheim. E. coli DNA-dependent RNA polymerase was purified as described (9) with the exception that DNA-agarose instead of DNA-cellulose chromatography was performed. recA protein was a generous gift from Drs. McEntee and Weinstock (Stanford). It was purified from E. coli KM 1862 as described by Weinstock et al. (10). It was more than 95% pure as determined by visual inspection of the patterns of electrophoresis on polyacrylamide gels in the presence of Na dodecyl sulfate, which was performed as described (11). The concentration of recA protein was determined assuming an extinction coefficient at 290 nm of ε 290 = 0.216. Antibodies against recA protein were obtained as described (8). The serum was precipitated in (NH4)2SO4 and the pellet was run on a mixed bed

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1 G. M. Weinstock, personal communication.
column formed with diethylamino cellulose and carboxymethyl cellulose. Typically, from 4 ml of serum, one obtains 220 mg of proteins of which about 90 mg precipitate in 30% (w/v) (NH₄)₂SO₄. Of these, about 40 mg are excluded from the mixed bed column and constitute the IgG pool. Electrophoretic analysis of the IgG on polyacrylamide- sodium dodecyl sulfate slab gels reveals a band of stainable material (~90% of the stain) migrating as E. coli RNA polymerase (factor (Mₒ = 95,000) and a diffuse minor band (10% of the stain) the mobility of which corresponds to Mₒ = 20,000. The IgG so purified are stable for more than 1 year at −70 °C in 20% Na phosphate, pH 7.5. The antibodies were labeled with ¹²⁵I as described (8), stored frozen at −20 °C, and thawed immediately before use. They can be used for the immunoradiometric assay for 2–3 weeks after labeling. After that period, background increase is observed and it is more difficult to achieve the reproducibility of the results.

Bacterial Growth and Analysis of recA Protein—Bacteria were grown in L broth at 37 °C with shaking. Bacterial growth was followed by measuring the optical density at 650 nm as well as the protein concentration in order to be able to determine the amount of recA protein per microgram of total proteins in the culture. This was done because the possible filamentation of the bacteria after chemical treatment does not allow a straightforward correlation between the number of colonies on a Petri dish and the number of viable cells in the culture broth. In our experimental conditions, the protein concentration of nontreated or mitomycin C (5 pg/ml)-treated wild type bacteria varies linearly with the optical density at 650 nm between 0.01 and 2.5 (see Fig. 1). The duplication time of the bacteria (AB1157) in the control was 25 min in L broth. Mitomycin C did not affect the rate of growth as measured by the increase of turbidity. When the bacterial culture reached the required concentration (A₅₀ = 0.2) 50 ml were withdrawn and added to 5 ml of prewarmed culture medium containing mitomycin C at different final concentrations. Controls with the solvent of the drug or with only water added were run in parallel.

At the indicated times after the induction, aliquots were withdrawn (1 to 5 ml) and centrifuged. The bacterial pellet was resuspended in IRMA-A or in IRMA-B (for total protein content determination), chilled in ice, and sonicated three times for 30 s (power, 50 watts, with a sonicator from the Branson Sonic Power Company, Danbury) and stored frozen at −70 °C (the effect of sonication was monitored by checking in the light microscope that no more bacteria were to be seen).

After thawing, portions of the sonicated extracts (5 to 100 µl) were assayed for total protein concentration and for recA protein according to the immunoradiometric assay (8). The protein concentration was determined according to the method of Lowry et al. (12).

Spheroplasts—Spheroplasts were prepared from AB1157 grown in minimal medium up to 10⁹ cells/ml following exactly the method described by Young and Sinsheimer (13) by lysozyme-EDTA-MgSO₄ treatment. The chemical to be tested for its efficacy in the induction of recA protein was added just after the addition of MgSO₄. The mixture was incubated at 37 °C and after the indicated times after the addition of the drug aliquots were taken, sonicated and stored at −70 °C until they were assayed for the content of recA protein. In these cases, the content of recA protein is expressed as micrograms of recA protein per ml of the incubation mixture, because the large amount of added lysozyme makes it difficult to detect any variation of the total protein content.

Bacterial Strains—The following bacterial strains were used in this study: AB 1157 (wild type for the functions of interest in the present work); AB 2500 (uvra); AB15821IF gal sra and uvra, with a big deletion in recA gene). These strains were a generous gift of Dr. George Weinstock.

RESULTS

The immunoradiometric assay used for the quantitative determination of recA protein can be summarized in the following steps. 1) Polystyrene tubes are coated with rabbit antibodies against recA protein. 2) recA protein or bacterial extracts to be tested for their recA protein content are incubated in the coated polystyrene tubes. 3) ¹²⁵I-labeled rabbit antibodies against recA protein are incubated in the coated polystyrene tubes. 4) The amount of radioactivity retained in the tubes is measured, and the amount of recA protein is determined using a standardization curve where the retained radioactivity is plotted versus the amount of pure recA protein added per coated tube.

The parameters which we have recognized as intervening in the assay are the following: (a) the time and temperature to be allowed to cold antibodies to adhere to the polystyrene test tubes; (b) the amount of cold IgG adhering to the test tube; (c) the effect of rinsing on the adhesion of the antibodies to the test tube; (d) the time and temperature of incubation of antigens in antibody-coated test tubes; (e) the time and temperature of incubation of labeled antibodies; (f) the per cent of IgG being antibodies against recA protein; (g) the concentration of labeled antibodies to be used in the assay; (h) the effect of the presence of monovalent cations, single stranded DNA and ATP, and the centrifugation of the samples after sonication; (i) the kind of test tubes to be coated; (j) the aging of coated IRMA tubes; (k) the origin of the serum.

Using ¹²⁵I-labeled antibodies to coat the polystyrene test tubes, no difference was detected in the amount of antibodies retained on the tubes, which could be attributed to the time allowed for coating (4 or 24 h at room temperature) or to the extent of rinsing (from one to three times with whirlmixing for 30 s). Plotting the fraction of retained antibodies versus the amount of added antibodies, one can conclude that half a microgram of total IgG is adsorbed on saturated polystyrene test tubes. Points (d) and (e) were explored by establishing standard curves after having varied these two parameters: the length of the time interval of incubation of antigen in antibody-coated test tubes and the time interval during which the labeled antibodies are allowed to incubate. The first parameter does not appear to be critical in the range from 1 to 4 h at 37 °C, while the second one is extremely important; 1 hour is clearly insufficient to allow the saturation of recA protein molecules with the hot antibodies, while incubation for 16 h appears to fulfill the requirements for obtaining a steep and reproducible standard curve.

The per cent of IgG being antibodies against recA protein was determined as follows: increasing amounts of pure recA protein (1 to 2000 ng) are added to coated polystyrene tubes as described above (series 1). After incubation and decanting, labeled antibodies are added and incubated overnight at room temperature, after which they are decanted but, instead of being discarded, they are added to tubes prepared with cold antibodies and 2000 ng of pure recA protein and allowed overnight at room temperature (series 2). The two series of tubes are then rinsed and counted. The plot of counts per min retained versus added recA protein levels off above 300 ng of recA protein. If all the antibodies against recA protein are

![Fig. 1. Plot of protein concentration versus optical density at 650 nm for AB1157 grown in L broth. The concentration is expressed as micrograms of protein per ml of bacterial culture. △, nontreated cultures; ■, mitomycin C-treated cultures.](image-url)
retained in the tube prepared with 2000 ng of recA protein in the first series of tubes, one expects that only background will be counted in the last tube of the second series. In this case, assuming that the IgG are homogeneously labeled, one can express the fraction of total IgG which are antibodies against recA protein as fraction of antibodies against recA protein = (cpm retained with 2000 ng of recA protein)/(total cpm added). From data obtained in this way it appears that IgG from different rabbits contain different relative amounts of antibodies against recA protein (from 2.5 to 10%). The optimal concentration of radioactive antibodies to be used in the assay was determined as described (8).

In order to rule out the possibility that the assay may be biased by the presence of single-stranded DNA, ATP, and salts (which might not be under the control of the experimenter, according to the conditions of growth of the bacteria and of sonication) we have mixed known amounts of purified recA protein with heat-denatured salmon sperm DNA and/or ATP at 0.5 M or 2 M NaCl and we have then performed the immunoradiometric assay. From these results, it appears that no difference is to be ascribed to the presence of either one of these substances. The centrifugation of the sonicated bacteria prior to immunoradiometric assay did not introduce differences in the results. We have been using several types of polystyrene test tubes (Falcon, LES, Dynatech Laboratories) with practically equivalent results. We have nevertheless observed that after having been stored for 3–4 months at −20 °C in the presence of IRMA-A buffer, the coated tubes yield a standard curve which is significantly less steep than the standard curve obtained with freshly coated test tubes.

We have observed that the range of linearity of the standard curve depends on the particular antibodies used. The standard curve published in our preliminary paper (8) was obtained with IgG from a rabbit the antibodies of which contained 10% of anti-recA protein antibodies. With the IgG pool from another rabbit which contained only 2.5% of antibodies against recA protein the standard curve was linear in the range of 0.1–2 ng of recA protein. The identity of the figures (10%, 10 ng; 2.5%, 2 ng) in the range of linearity of the assay may be purely coincidental.

The standard curve does not change when the purified recA protein is incubated in the presence of portions of crude extracts of a deletion mutant (159Δ21) which lacks endogenous recA protein. Moreover, the data obtained are identical (within ±20%) when different amounts of bacterial extracts are added at concentrations of 7.3, 73, and 367 ng/ml, thus indicating that E. coli proteins other than recA protein play a little role (if any) in the binding of antibodies anti-recA protein.

When different concentrations of extracts from bacteria are added instead of purified recA protein, one observes a linear variation of retained radioactivity versus the amount of bacterial extract. This result indicates that, in the explored concentration range, no variation in the antibody-binding activity is to be ascribed to effectors or inhibitors and it allows one to measure recA protein in different volumes and thus reduce the indeterminacy due to background variations: four or five different volumes of bacterial extract are assayed per sample; the counts per min retained are plotted against the assayed volume. The slope of the curve is expressed as counts per min/μl and it is compared to a standard curve (cpm/ng of recA protein) to yield the concentration of recA protein in the sample.

**Effect of Mitomycin C on the Content of recA Protein in Bacteria**—The immunoradiometric assay has been used to measure the amount of recA protein in E. coli at different stages of growth after treatment of the bacteria with different concentrations of mitomycin C. No effect on the rate of increase of turbidity was observed in the bacterial cultures treated with the drug.

The kinetics of induction of recA protein after addition of mitomycin C to exponentially growing AB1157 and AB2500 (uvrA) is reported in Figs. 2 and 3. It can be seen that after a short lag the recA protein content in AB1157 increases, reaches a maximum 60 to 90 min after the addition of the drug and, then it decreases to lower values.

Both the lag and the time interval necessary to reach the maximum depend on the dose of mitomycin C. It is not known whether the decrease is a consequence of the metabolism of the drug or of the dilution of the drug among the total proteins of the growing, filamenting bacteria. In the control, no difference is observed in the basal level of recA protein nor on the rate of growth when L broth or L broth plus the solvent of mitomycin C (20 mM sodium acetate buffer, pH 5) is added to the bacteria.

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**Fig. 2.** Kinetic analysis of the dose-response effect of mitomycin C on E. coli AB1157 bacteria at ΔA600nm = 0.2 were mixed with mitomycin at t = 0. Mitomycin concentration: ○, 0.15 μg/ml; □, 1 μg/ml; Δ, 5 μg/ml; ▲, 10 μg/ml.

**Fig. 3.** Kinetic analysis of the dose-response effect of mitomycin C on E. coli AB2500 (uvrA). Bacteria at ΔA600nm = 0.4 were mixed with mitomycin C at t = 0. Mitomycin concentration: ○, 0.15 μg/ml; □, 1 μg/ml; Δ, 5 μg/ml; ▲, 10 μg/ml.
The content of recA protein in the control is not a constant; it appears in the majority of the cases to reach a maximum 20 to 40 min after the bacterial culture has reached A_{600} = 0.2 and then it drops. It is not known whether this small peak of recA protein is significant from the point of view of the variation of the basal level of recA protein. We have observed it in six out of nine control curves, and we have decided to see what happens if the cells are induced when they are at that stage of growth. We have observed that when the cells are induced with mitomycin C 45 min after the culture has reached A_{600} = 0.2 (i.e., at an A_{600} ~ 0.7), the lag period is much longer (up to an hour) but the maximum level of induction is the same (data not shown).

The effect of mitomycin C on the synthesis of recA protein in a uvrA mutant (strain AB2500) is reported in Fig. 3. Since the rate of increase of the turbidity of such a bacterial culture (apparent duplication time, 40 min) was not affected by the presence of the drug, we checked its phenotype to make sure that it corresponds to what is expected for an excision repair deficient strain. It turned out that a UV flux of 2.4 J per m² allowed only 26% of survival, while for a similar survival the AB1157 strain had to be irradiated with a UV flux greater than 80 J/m².

The relevant aspect of the data reported in Fig. 3 is that the maximum level of the content of recA protein in this strain is less than half the maximum level in the wild type. Moreover, after the maximum level is reached, the relative content of recA protein remains constant, as it is expected for an excision repair-deficient strain because one can imagine that the DNA damage provides a persistent signal for the induction of recA protein.

The data reported in Figs. 2 and 3 were obtained allowing the drug to remain in the presence of the bacteria throughout the experiment. In order to avoid the possibility that these results might be biased by the continuous formation of mitomycin C adducts on bacterial DNA, we have grown the cells up to A_{600} = 0.2, and we have then added the drug and allowed 30 min at 37 °C with shaking. After centrifugation and resuspension in prewarmed fresh medium, we have withdrawn aliquots at different times and we have proceeded to the immunoradiometric assay. The results of such an experiment are very similar to the results reported in Figs. 2 and 3. The uvrA mutant continues to produce high levels of recA protein for 2 h after the induction, while in the wild type the recA protein reaches a maximum 90 min after the induction and then it drops with a half-time of 20 min. No survivors were found after plating on LB agar for the mitomycin C-treated uvrA mutant, while the treated wild type survivors corresponded to a few per cent of the control (data not shown).

**Induction of recA Protein in Spheroplasts**—When exponentially growing bacteria are treated with EDTA-lysozyme and MgSO₄, in order to obtain spheroplasts, one observes a 3-fold increase of the level of recA protein. This phenomenon is observed when bacteria are grown in minimal medium as well as in rich medium. Addition of mitomycin C (5 μg/ml) or of nalidixic acid (40 μg/ml) to the spheroplasts promotes a striking increase of the total content of recA protein in the suspension. The content levels off 80 to 100 min after the addition of the drugs. These results are reported in Fig. 4.

These spheroplasts are satisfactorily stable. As a matter of fact, incubation of the spheroplasts at 0 °C for up to an hour before the addition of the drugs does not affect their competence to produce recA protein for the next 2 h, up to the same maximum level as the one reached in Fig. 4 (data not shown).

In order to perform a quantitative comparison of the data in Fig. 4 with the data in Fig. 2, one has to keep in mind that the spheroplast concentration corresponds to the one of a culture having reached A_{600} = 0.8 (~2 × 10⁸ cells per ml) because bacteria grown to an A_{600} = 0.4 were pelleted and resuspended in half the initial volume. Thus, if one assumes that no relevant total protein synthesis has occurred after plasmolysis of the bacteria, the maximum relative level of recA protein turns out to be 1 ng per μg of total protein.

This is roughly twice less than the maximum level reached in Fig. 2, but the disagreement will appear less striking by taking into account the fact that upon counting the spheroplasts under the light microscope one finds 10⁸ bodies per ml, i.e., one can consider that half the bacterial population has been lysed and is no longer competent for synthesizing recA protein.

**DISCUSSION**

The immunoradiometric assay recently set up in our laboratory (8) has been used for the quantitative determination of the content of recA protein in E. coli. This assay is sensitive; it has a few drawbacks and some advantages which deserve being discussed.

First, the ¹²⁵I-labeled antibodies can be used for only a short period of time after labeling (2–3 weeks at most). So, in order to use freshly labeled antibodies yielding low background and steep standard curves without being obliged to undergo the labeling procedure too often, one has to prepare a number of samples and to store them at ~70 °C, before assaying them all together. This slows down the process of selecting the best conditions for an experiment and the choice of the questions to be asked.

Second, random variations affect the sample volume to be assayed, the IgG-coated test tube, and the radioactivity to be counted. In order to reduce the effect of this source of experimental indeterminacy, one has to take different volumes of each sample, plot the count per min retained versus the added volume, and determine the concentration of recA protein using a standard curve. The short mean life of ¹²⁵I makes it necessary to redetermine the standard curve at every series of samples to be counted. We estimated that the experimental measurements of recA protein are affected by an indeterminacy which might be as large as 30%, in particular for determinations of relative values lower than 0.2 ng of recA protein per μg of total protein. In order to reduce this indeterminacy to acceptable levels, one should measure several times the
same different volumes from the same sample. These two drawbacks are compensated by the long life of the purified IgG, by the extremely small consumption of antibodies in the assay, by the ease of radio-labeling, and by the more than acceptable reproducibility of the results.

Exponentially growing or plasmolyzed bacteria were treated with mitomycin C or nalidixic acid, and the relative amount of recA protein was determined using the immunoradiometric assay. From the data reported, it appears that in mitomycin C-treated wild type bacteria, the relative amount of recA protein increases 15-fold within 60 min and then it decreases to nearly basal level values. On the other hand, in an excision repair deficient mutant (uvrA), mitomycin C treatment promotes a similar increase of the recA protein content, but no decrease is observed for up to 2 h after induction.

These results seem to agree with the hypothesis that persistent DNA deformation or blockage of the growing replicative fork constitutes the "signal" for the derepression of the recA gene since mitomycin C is known to form cross-links between the two strands of the DNA. On the other hand these results do not appear to agree with another model proposed for the derepression of recA gene, according to which the excised single-stranded DNA constitutes the "signal." (According to this model, the single-stranded DNA fragment excised in order to eliminate the DNA damage interacts with the recA protein present at the basal level, confers to it a proteolytic activity, and makes it able to cleave the product of lexA gene, which is the repressor of recA gene.) As a matter of fact, a naive analysis of the results obtained with the uvrA mutant could lead one to think that an excision repair-deficient mutant will not be able to produce fragments of single-stranded DNA. Thus in an uvrA mutant treated with mitomycin C, this model does not predict the observed increase of the relative amount of recA protein nor its maintenance at high levels for long time.

Upon treatment of wild type cells with lysozyme and EDTA, a striking and extremely reproducible 3-fold increase of the relative content of recA protein is observed. Such spheroplasts are competent for the synthesis of large amounts of recA protein upon treatment with mitomycin C (which is supposed to block DNA replication by formation of interstrand cross-links) or with nalidixic acid (which is believed to stop DNA replication by action on DNA gyrase). Since the spheroplasts are competent for the replication of DNA, one is let to ask what is the "signal" for the 3-fold stimulation of the level of recA protein. Since we have no evidences of DNA damages being generated by plasmolysis of the bacteria, a naive analysis, resting mainly on our ignorance of molecular phenomena within the cells, could lead one to think that the action of the lysozyme on the bacterial wall leads to a conformational change of the bacterial chromosome and that this deformation constitutes the signal for the derepression of recA gene. Nevertheless, we do not have evidence against the possibility that some recA protein bound to the bacterial wall and membrane might be made "detectable" to the immunooassay through the lysozyme treatment or that this treatment affects some other physiologically important aspects of the cell, which might in turn induce the synthesis of recA protein.

An extremely interesting aspect of the radioimmunooassay connected with the competence of spheroplasts to be stimulated by classical inducers of recA protein is that they can be used to test those factors believed to induce the inducible DNA repair functions, such as single-stranded oligonucleotides, DNA intercalating drugs, and free radicals. Such experiments are currently undertaken in our laboratory.

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REFERENCES