

***recF*-dependent induction of *recA* synthesis by coumermycin, a specific inhibitor of the B subunit of DNA gyrase**

(SOS functions/topoisomerase/novobiocin/DNA repair)

CASSANDRA L. SMITH

Department of Human Genetics and Development, College of Physicians and Surgeons of Columbia University, 701 West 168 Street, New York, New York 10032

Communicated by Gilbert Stork, January 27, 1983

ABSTRACT Genetic and biochemical studies on enzymes known to be involved in regulating DNA supercoiling yield a complex spectrum of effects on the *Escherichia coli* SOS system. Previous studies indicated that only inhibition of DNA gyrase by antibiotics that act on the DNA gyrase A subunit results in turning on the *E. coli* SOS system. Here we show that coumermycin, an antibiotic that acts on the DNA gyrase B subunit, can also induce. Like nalidixic acid induction, coumermycin induction is dependent on the *recBC* DNase. In both cases induction apparently results from a response of the cell to the DNA gyrase-inhibitor complex rather than just the loss of DNA gyrase activity. However, unlike induction by the DNA gyrase A-specific antibiotics, coumermycin induction also requires the *recF* gene product. This demonstrates a functional relationship between DNA gyrase and the *recF* gene product.

DNA gyrase catalyzes the introduction of negative superhelical twists into double-stranded closed-circular DNA in an ATP-dependent reaction (1); in the absence of ATP, gyrase relaxes supercoiled DNA (for reviews see refs. 2 and 3). The enzyme is composed of two subunits, originally identified as targets for two types of antibiotics. The DNA gyrase A subunit is the target of nalidixic and oxolinic acid (4, 5) and has the breakage/rejoining activity of the enzyme. In the presence of nalidixic acid DNA gyrase forms a relaxation complex with DNA. The DNA gyrase B subunit mediates energy transduction required in the supercoiling reaction and is the target for novobiocin and coumermycin A1 (6). Inhibition of DNA gyrase by any of these antibiotics threatens the survival of *Escherichia coli* because DNA synthesis is arrested (7, 8).

A halt in DNA synthesis leads to the coordinated induction of numerous *recA*-dependent phenomena, termed SOS functions (for a recent review see ref. 9), which promote the survival of *E. coli* and its phages. SOS functions include an error-prone DNA repair, prophage induction, and inhibition of *recBC* DNase-mediated DNA degradation. The induction of these phenomena depends on the activation of the constitutively made *recA* protein to act as a protease and cleave its own repressor (10) and other repressors (11, 12).

One would expect that interference with DNA gyrase would lead to turning on the SOS system. In fact, treatment of *E. coli* with nalidixic or oxolinic acid (gyrase A-specific antibiotics) leads to the immediate turn-on of the SOS system (13). However, we did not observe turn-on by novobiocin, a gyrase B-specific antibiotic (14), and others have reported no SOS induction when *E. coli* was treated with other gyrase B-specific antibiotics (coumermycin and chlorobiocin) or when a gyrase B temperature-sensitive mutant was incubated at the restrictive temperature (15, 16). Here, we have reexamined this discrepancy by using

Table 1. *E. coli* strains used

Strain	Relevant genotype*	Source
AB1157	Wild type	M. Oishi
CS2369	<i>gyrB</i> ⁻ (<i>cou</i> ^R)	This study†
JC9239	<i>recF</i> ⁻	A. J. Clark
MO1518	<i>recB</i> ⁻	M. Oishi
JC8679	<i>recB</i> ⁻ <i>recC</i> ⁻ <i>sbca</i> ⁻	R. Kolodner

* All strains are derivatives of AB1157 and contain the following genetic markers: *thr-1*, *leu-6*, *lacY1*, *galK2*, *ara-14*, *xyl-5*, *mtl-1*, *proA2*, *his*, *argE3*, *str-31*, *tsx-33*, and *supE44*.

† Constructed by phage P1 transduction of the *gyrB*⁻ (coumermycin resistance) mutation from *E. coli* strain NI748, obtained from N. R. Cozzarelli, to AB1157.

a more sensitive assay for *recA* induction that we have developed.

MATERIALS AND METHODS

Bacterial strains used in this study are listed in Table 1. All experiments were carried out with *E. coli* cells grown at 37°C with shaking in supplemented minimal medium, containing ampicillin at 25 µg/ml. Details of procedures used for induction assays can be found in ref. 16. Data are plotted as anthranilate synthase (ASase) activity per ml of culture. However, specific activities parallel these total activity values. The relative merits of different ways of plotting these data will be discussed elsewhere (16).

To follow DNA synthesis cells were grown at 37°C with shaking in supplemented minimal medium containing ampicillin at 25 µg/ml and thymidine at 1 µg/ml to a density of 1×10^8 cells per ml. At that time thymidine was added to give an additional 5 µg/ml along with deoxyadenosine at 200 µg/ml and [³H]thymidine [45 Ci/mmol; Amersham (1 Ci = 3.7×10^{10} Bq)] at 2 µCi/ml and the incubation was continued. Twelve minutes later portions of the culture were treated with the various DNA gyrase inhibitors.

All antibiotic solutions were stored at 4°C. Nalidixic acid and novobiocin were obtained from Sigma. Oxolinic acid was a gift from Warner-Lambert (Ann Arbor, MI). Nalidixic acid and oxolinic acid were dissolved in 50 mM NaOH at 10 and 1 mg/ml, respectively. Novobiocin was made up at 10 mg/ml in water.

Coumermycin was obtained from Bristol Laboratories (Syracuse, NY) or purchased from Godfrey Science and Design (Syracuse, NY) and dissolved in dimethyl sulfoxide (Me₂SO) at 5 mg/ml. Different batches obtained over a 5-year period behaved identically. Because coumermycin is typically used at 20 µg/ml, use of a Me₂SO stock solution results in a final Me₂SO concentration of 0.4%. Me₂SO alone at this concentration has no effect on the constitutive activity of the *recA-trp* gene fu-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ASase, anthranilate synthase; Me₂SO, dimethyl sulfoxide.

sion. Me_2SO alone is not an inducer. We examined the effect of Me_2SO concentrations up to 2.5% on the activity of various inducing agents. No effect was seen except for coumermycin, for which a progressive inhibition of induction activity is seen as the Me_2SO concentration is increased. The kinetics of coumermycin induction are unaltered by Me_2SO , but the inhibition may explain why others in the past have been unable to observe coumermycin induction. It is possible to use aqueous stock solutions of coumermycin, but these are unstable to prolonged storage.

RESULTS

Earlier studies of *recA* induction have relied mainly on tedious indirect assays. To circumvent the difficulties associated with these systems we constructed a gene fusion between the *recA* control region and the *trpED* genes on pBR322 derivative plasmid, pCS16. The fusion region of pCS16 is shown in Fig. 1. This system closely mimics the known behavior of the *recA* operon *in vivo* (unpublished data). SOS induction is monitored by assaying the production of ASase, the product of the *trpED* genes. Different strains carrying pCS16 show 2- to 4-fold variation in ASase activity. These differences are not significant for any of the results described below.

The *recA-trp*-containing plasmid, pCS16, was used to transform the various *E. coli* strains listed in Table 1 and SOS inducibility by various DNA gyrase inhibitors was measured. When we examined the inducibility of *recA* with DNA gyrase inhibitors in a wild-type *E. coli* strain the expected gyrase A-dependent induction of *recA* was observed. In addition we found that coumermycin A1, a gyrase B-specific antibiotic, also turned on *recA* synthesis (Fig. 2A). The kinetics of turn-on with coumermycin were distinctly slower than that observed with gyrase A-specific antibiotics and other inducers. This is not due to a delay in uptake of the drug or to the speed at which DNA synthesis is turned off. We find coumermycin, just like gyrase A-specific antibiotics, is able to turn off DNA synthesis within 5 min after addition to *E. coli* cultures (Fig. 3).

It is not clear why others in the past have been unable to observe coumermycin induction. The relatively weak response and slow kinetics, while easily detected by our fusion technique, may have been difficult to see by less sensitive techniques. Other possible explanations are discussed in *Materials and Methods*.

It was disturbing that a typical dose of coumermycin (20 $\mu\text{g}/\text{ml}$) induced *recA* synthesis, whereas no induction was observed with typical doses (100–300 $\mu\text{g}/\text{ml}$) of novobiocin (Fig. 2). This, plus the fact that temperature-sensitive gyrase B mutants failed to show induction at the restrictive temperature (15), raised the possibility that SOS induction by coumermycin is not due to its action on gyrase B. However, no *recA* induction was observed with two different, proven gyrase B coumermycin-resistant mutants. Results obtained with one of these mutants are shown in Fig. 2B. This means coumermycin-mediated *recA* induction must be acting via the gyrase B subunit. Coumermycin induction of *recA* does not occur in *recA*⁻ or *lexA*⁻ strains (data not shown). Thus, presumably coumermycin induction occurs

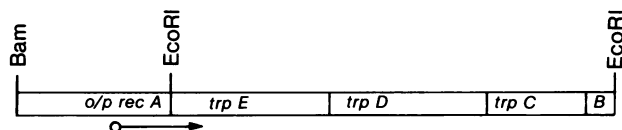


FIG. 1. A fusion between the *E. coli* *recA* operon and *trpED* genes. The *Bam*HI/*Eco*RI DNA fragment shown is cloned in pBR322. o/p, Operator-promoter.

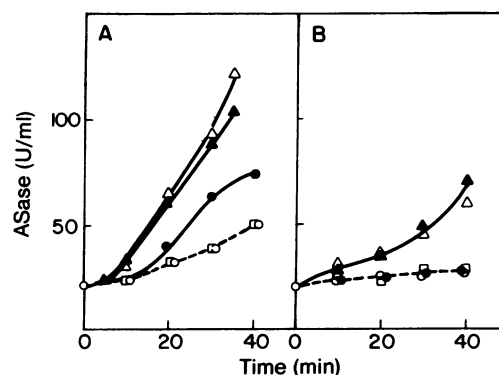


FIG. 2. Induction of *recA*-directed ASase synthesis in wild-type and *gyrB*⁻ (coumermycin-resistant) *E. coli*. One unit (U) of ASase catalyzes formation of 1 nmol of anthranilic acid per 10 min at 37°C. *E. coli* strain AB1157 (A) and CS2369 (B) were grown to 1×10^8 cells per ml and treated with coumermycin at 20 $\mu\text{g}/\text{ml}$ (●), nalidixic acid at 40 $\mu\text{g}/\text{ml}$ (▲), oxolinic acid at 5 $\mu\text{g}/\text{ml}$ (△), or novobiocin at 100 $\mu\text{g}/\text{ml}$ (□). ○, Untreated control.

by normal activation of *recA* proteolytic activity.

Why is there no SOS induction with typical novobiocin doses? We examined the effect of typical novobiocin doses on the ability of other drugs to induce *recA*. In every case roughly 2-fold inhibition of *recA* synthesis was observed. Similar inhibition is observed with the other DNA gyrase inhibitors in genetic backgrounds where they themselves are not inducers. Such a partial inhibition implies that gyrase activity is involved (but not required) in some stage of *recA* expression.

Guided by the observations of R. Menzel and M. Gellert that very high doses of novobiocin induce *recA* protein (personal communication), we studied the effect of novobiocin in our system at concentrations all the way from 100 $\mu\text{g}/\text{ml}$ to 2.5 mg/ml. Around 1 mg/ml novobiocin a small amount of *recA* induction is observed. This shows that failure of typical novobiocin doses to induce is due not to some qualitative difference between novobiocin and coumermycin but merely to the fact that novobiocin inhibition of *recA* synthesis masked its induction. Presumably with coumermycin and other stronger gyrase inhibitors induction is so rapid and pronounced that the simultaneous inhibition goes unnoticed.

One is still left with the puzzle that inhibition by gyrase A- or gyrase B-specific antibiotics leads to *recA* induction, whereas no induction was seen in a strain with temperature-sensitive gyrase B (15). To explore this point further we examined an ad-

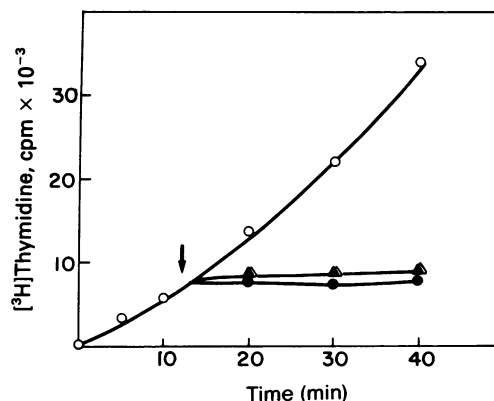


FIG. 3. Inhibition of DNA synthesis by antibiotics that act on DNA gyrase. *E. coli* strain AB1157 was grown to 1×10^8 cells per ml and treated (arrow) with coumermycin at 20 $\mu\text{g}/\text{ml}$ (●), nalidixic acid at 40 $\mu\text{g}/\text{ml}$ (▲), or oxolinic acid at 5 $\mu\text{g}/\text{ml}$ (△). ○, Untreated control.

ditional temperature-sensitive gyrase B mutant (17) and a temperature-sensitive gyrase A mutant (18), using our sensitive assay. No induction was seen in either case, even after prolonged incubation at the restrictive temperature. These results imply that elimination of gyrase activity is insufficient to cause induction of *recA*. Some property of gyrase-inhibitor complexes, perhaps their inability to dissociate from DNA, must be responsible for generating an induction signal. In agreement with previous results (15), we observe the curious fact that gyrase-specific antibiotics were still able to induce in temperature-sensitive gyrase mutants at the restrictive temperature. This may reflect stabilization of gyrase by the bound antibiotics or simply that thermal inactivation of gyrase activity leaves unaltered the property of gyrase-inhibitor complexes required for induction.

We tested the involvement of various recombination pathways (19) on *recA* induction mediated by gyrase-antibiotic complexes. As had been reported previously (13), nalidixic acid-mediated SOS induction is not seen in *recB⁻ recC⁻* cells (Fig. 4A). Similarly, coumermycin-mediated induction is also not seen (Fig. 4A). Recombinational proficiency can be restored to *recB⁻ recC⁻* cells by either the introduction of the phage λ recombination system or nonallelic suppressor (*sbcB⁻* or *sbcA⁻*) mutations. These are known to restore SOS induction by nalidixic acid in *recB⁻ recC⁻* cells (20, 21). The results shown in Fig. 4B indicate that, when cells lacking the *recBC* DNase carry an additional *sbcA⁻* mutation, SOS induction by both coumermycin and nalidixic acid is once again observed.

Although the genetic manipulations described above restore the recombinational proficiency of *recB⁻ recC⁻* cells, they do not restore *recBC* DNase activity. Apparently, the role of that enzyme can be replaced by λ exonuclease or, in *sbcA⁻* cells, by exonuclease VIII (22). Both of these nucleases are double-strand 5'-3' exonucleases. Presumably it is this activity of the *recBC* DNase that is important in turning on the *E. coli* SOS system after both gyrase A and gyrase B inhibition. The situation must be somewhat different in *recB⁻ recC⁻ sbcB⁻* cells. Here the loss of exonuclease I, a single-strand exonuclease, is believed to channel recombination intermediates into the *recF* recombinational pathway. We cannot examine *recA* induction with pCS16 in *recB⁻ recC⁻ sbcB⁻* cells because they are unable to maintain plasmids (23). Instead we chose to examine the *recF* dependence of induction by gyrase inhibitors.

Coumermycin induction is not seen in *recF⁻* cells (Fig. 5), even when these cells are studied for extended periods of time. In contrast, nalidixic acid induction is unaffected by *recF⁻* mutations. The specific requirement for the *recF* gene product in SOS induction by a gyrase B but not a gyrase A inhibitor implies that the *recF* gene product has a unique interaction with gyrase B-coumermycin complexes. A *recA* induction that is totally dependent on the *recF* gene product has not been reported pre-

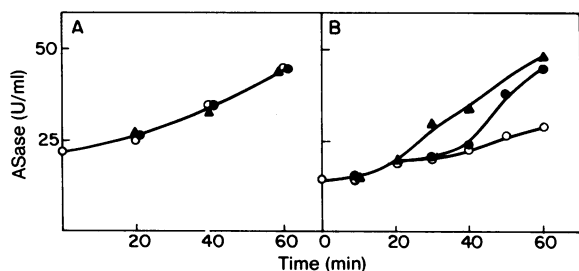


FIG. 4. Induction of *recA*-directed ASase synthesis in *E. coli* *recB⁻ recC⁻* and *recB⁻ recC⁻ sbcB⁻* strains. *E. coli* strains MO1518 (A) and JC8679 (B) were grown to 1×10^8 cells per ml and treated with coumermycin at 20 μ g/ml (●) or nalidixic acid at 40 μ g/ml (▲). ○, Untreated control.

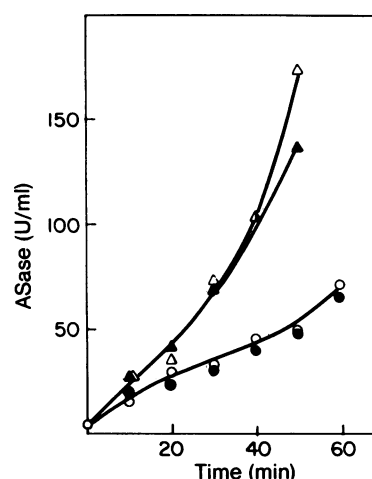


FIG. 5. Induction of *recA*-directed ASase synthesis in an *E. coli* *recF⁻* strain. *E. coli* strain JC9239 was grown to 5×10^7 cells per ml and treated with coumermycin at 20 μ g/ml (●), nalidixic acid at 40 μ g/ml (▲), or oxolinic acid at 5 μ g/ml (Δ). ○, Untreated control.

viously. The *recF* recombination pathway is considered to be a minor pathway that normally functions only in the absence of exonuclease I [*sbcB* gene product (24, 25)]. However, the *recF* pathway appears to involve unique recombination intermediates; the importance of these needs to be assessed (26, 27). In addition, *recF⁻* cells are sensitive to ultraviolet irradiation and there is evidence that the as-yet-unidentified *recF* gene product plays a role in DNA repair (28). The association of *gyrB* and *recF* may provide a key to an essential role of the *recF* gene product in DNA repair.

DISCUSSION

Changes in supercoiling are known to affect expression of various operons by altering transcription (29-32). Coumermycin induction is not simply due to a direct effect of the drug on transcription because of the *recA*, *recBC*, and *recF* dependence. In addition, all the DNA gyrase inhibitors partially inhibit *recA* expression when they are added to cells in which they do not induce. This indicates that changes in supercoiling caused by DNA gyrase inhibition result in down rather than up expression of the *recA* operon. To induce the *recA* operon some specific property of gyrase-inhibitor complexes is required and not simply inhibition of gyrase activity.

The very different kinetics and *recF* dependence of *recA* turn-on resulting from gyrase A- and gyrase B-specific antibiotics imply different induction mechanisms. In fact, when *E. coli* is treated with gyrase A- or gyrase B-specific antibiotics very different things happen. In the former case, DNA replication is rapidly inhibited (33) and the chromosome is rendered susceptible to cleavage by protein denaturants, as in the behavior of relaxation complexes (34). Drlica and colleagues (35) have argued that the rapid halt of DNA synthesis is caused by inhibition of gyrase molecules associated with replication forks rather than those that appear to be distributed at intervals over the chromosome. Nalidixic acid induction of the SOS system is rapid and is almost certainly related to tight drug-gyrase-DNA complexes. However, it is not clear whether all gyrase-antibiotic complexes lead to induction or just those associated with replication forks.

Treatment of *E. coli* with gyrase B-specific antibiotics results in relaxation of the chromosome even when DNA replication is absent (36). This relaxation appears to be associated with the inhibition of gyrase molecules distributed over the chromo-

some. Coumermycin treatment results in a slow decrease in the rate of DNA synthesis, which reaches a plateau after 20 min. This decrease parallels the loss in superhelical density. However, when we followed the coumermycin inhibition of total DNA synthesis by continuous labeling (see Fig. 3) we did not see the slow turn-off reported by Drlica and Snyder (36). They measured the rate of DNA synthesis during a short labeling pulse, whereas we measured the amount of stable DNA synthesized. Thus the slowly decreasing DNA synthesis rate either is due to a DNA repair synthesis or represents continual initiation and rapid degradation of newly synthesized DNA. Coumermycin induction of *recA* shows the same kinetics as relaxation and the loss of this transient DNA synthesis.

Coumermycin-gyrase complexes do not render DNA susceptible to breakage. Why, then, does coumermycin induce, and are all gyrase molecules potential targets for coumermycin induction or just those associated with replication forks? One possibility is that the motion of a replication fork is arrested either by direct inhibition of gyrase there or by the first coumermycin-gyrase complex the fork reaches. Transient DNA synthesis and degradation at the stopped fork eventually result in generation of an induction signal. Alternatively, gyrase-antibiotic complexes distributed over the chromosome may be recognized directly by other proteins, such as repair enzymes, whose action results in DNA damage.

The *recF* dependence of coumermycin induction ought to be the clue that allows the induction mechanism to be sorted out. However, the absence of a proven function for *recF* is a serious impediment. In *E. coli* the *gyrB*, *recF*, *dnaN*, and *dnaA* genes are located in adjacent positions on the *E. coli* chromosome (37). The *dnaA* gene product is involved in replication initiation, and *dnaA* mutants are hypersensitive to gyrase inhibitors (38). In fact, it has been suggested that the close association of the *recF* gene with genes known to be involved in DNA replication and recombination implies a similar function for the *recF* gene product (39). It is tempting to speculate that, like other genes located in adjacent positions, the products of *gyrB*, *recF*, *dnaN*, and *dnaA* actually form a functionally significant multiprotein complex. Coumermycin inhibition of this target might then be the step leading to *recA* induction.

I thank Charles Cantor and Martin Gellert for helpful discussions and reading the manuscript and Esther Siegfried and Peter Ruvolo for excellent technical assistance. This work was supported by a U.S. Public Health Service Grant GM26103.

1. Gellert, M., Mizuuchi, K., O'Dea, M. H. & Nash, H. A. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3872-3876.
2. Gellert, M. (1981) *Annu. Rev. Biochem.* **50**, 879-952.
3. Cozzarelli, N. R. (1980) *Science* **207**, 953-960.
4. Sugino, A., Peebles, C. L., Kreuzer, K. N. & Cozzarelli, N. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4767-4771.
5. Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T. & Tomizawa, J.-i. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4772-4776.
6. Gellert, M., O'Dea, M. H., Itoh, T. & Tomizawa, J.-i. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4474-4478.
7. Crumplin, G. C. & Smith, J. T. (1976) *Nature (London)* **260**, 643-645.
8. Pisetsky, H., Berkower, I., Wickner, R. & Hurwitz, J. (1972) *J. Mol. Biol.* **71**, 557-571.
9. Little, J. W. & Mount, D. W. (1982) *Cell* **29**, 11-15.
10. Roberts, J. W., Roberts, C. W. & Craig, N. L. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 4714-4718.
11. Little, J. W., Mount, D. W. & Yanisch-Perron, C. R. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4199-4203.
12. Phizicky, E. M. & Roberts, J. W. (1981) *Cell* **25**, 259-264.
13. Gudas, L. J. & Pardee, A. B. (1976) *J. Mol. Biol.* **101**, 459-477.
14. Smith, C. L. & Oishi, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1657-1661.
15. Herrero, E., Orr, E. & Holland, I. B. (1981) *FEMS Microbiol. Lett.* **10**, 369-371.
16. Fairweather, N. F., Orr, E. & Holland, I. B. (1980) *J. Bacteriol.* **142**, 153-161.
17. Gellert, M., Fisher, L. M. & O'Dea, M. H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6289-6293.
18. Kreuzer, K. N. & Cozzarelli, N. R. (1979) *J. Bacteriol.* **140**, 424-435.
19. Clark, A. J. (1973) *Annu. Rev. Genet.* **7**, 67-105.
20. Crowl, R. M., Ahmed, S. U. & Boyce, R. P. (1978) *Nature (London)* **275**, 71-72.
21. Karu, A. E. & Belk, E. D. (1982) *Mol. Gen. Genet.* **185**, 275-281.
22. Barbour, D. S. & Clark, A. J. (1970) *Proc. Natl. Acad. Sci. USA* **67**, 955-961.
23. Vapnek, D., Alton, N. K., Basset, C. L. & Kushner, S. R. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3492-3496.
24. Kushner, S. R., Nagaishi, H., Templin, A. & Clark, A. J. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 824-832.
25. Horii, A. & Clark, A. J. (1973) *J. Mol. Biol.* **80**, 327-344.
26. Armengod, M.-E. (1981) *Mol. Gen. Genet.* **181**, 497-504.
27. Mahajan, S. K. & Datta, A. R. (1977) *Nature (London)* **14**, 652-653.
28. Rothman, R. H. & Clark, A. J. (1977) *Mol. Gen. Genet.* **155**, 279-286.
29. Botchan, P., Wang, J. C. & Echols, H. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3077-3081.
30. Smith, C. L., Kubo, M. & Imamoto, F. (1978) *Nature (London)* **275**, 420-423.
31. Yang, H., Heller, K., Gellert, M. & Zubay, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3304-3308.
32. Sanzey, B. (1979) *J. Bacteriol.* **138**, 40-47.
33. Engle, E. C., Manes, S. H. & Drlica, K. (1982) *J. Bacteriol.* **149**, 92-98.
34. Snyder, M. & Drlica, K. (1979) *J. Mol. Biol.* **131**, 287-302.
35. Drlica, K., Engle, E. C. & Manes, S. H. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6879-6883.
36. Drlica, K. & Snyder, M. (1978) *J. Mol. Biol.* **120**, 145-154.
37. Ream, L. W., Margossian, L. & Clark, A. J. (1980) *Mol. Gen. Genet.* **180**, 115-121.
38. Filutowicz, M. (1980) *Mol. Gen. Genet.* **177**, 301-307.
39. Clark, A. J. (1980) in *Mechanistic Studies of DNA Replication and Genetic Recombination*, ICN-UCLA Symposia on Molecular and Cellular Biology, eds. Alberts, B. & Fox, C. F. (Academic, New York), Vol. 19, pp. 891-899.