PROTEIN SYNTHESIS IN MINICELLS: POLYPEPTIDES ENCODED BY RECOMBINANT PLASMIDS CARRYING THE *E. coli lexA* GENE

by

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN MOLECULAR BIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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David Mount
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Date

To My Family

ACKNOWLEDGMENTS

I thank my advisor David Mount for his help and guidance during the course of this work, John Little for providing the plasmid strains used in this work and for helpful discussions, and Brian Knoll who has been a good friend as well as a scientific colleague.

My special thanks to my family for the years of patience and encouragement from my mother and my two brothers, and for the encouragement and practical help from my husband Don during the past three years.

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ABSTRACT

The protein products of recombinant plasmids carrying various alleles of the E. coli lexA gene were examined using minicells. The plasmid carrying the mutant lexA3 allele directed the synthesis of the 24,000 dalton lexA3 protein, and the plasmid carrying the wild type allele directed the synthesis of the 22,000 dalton protein believed to be the $lexA^+$ product. Both the wild type and the lexA3 protein were made in small amounts. The products of plasmids carrying three different amber mutations in the lexA3 gene were examined. In each case the lexA3 protein was not synthesized; and a new polypeptide, smaller than the lexA3 protein, was made. The new polypeptides, apparently amber fragments of the lexA3 protein, were greatly over-produced relative to the lexA3 protein. A plasmid carrying a new mutation causing reduced lexA function, isolated following mutagenesis of the $lexA^+$ plasmid, was found to direct the synthesis of a large amount of the putative $lexA^+$ protein in minicells. These results suggest that the lexA protein participates in the negative regulation of lexA gene expression.

CHAPTER 1

INTRODUCTION

Bacterial minicells are small anucleate cells which result from abnormal cell division in which the septum forms in the polar region of a rod shaped bacterium. While the occurrence of minicells is very rare in cultures of normal cells, mutant strains which produce large numbers of minicells have been isolated. Minicells can be purified from a culture on the basis of their small size and nearly spherical shape. Purified minicells contain normal amounts of RNA and protein, but are deficient in DNA, and a number of DNA associated enzymes such as DNA-dependent RNA-polymerase. Although they are unable to grow, they carry out a number of processes such as respiration and transport, serve as recipients in bacterial conjugation and support the growth of certain bacteriophages (for review see Frazer and Curtiss 1975).

Minicells purified from plasmid bearing strains have been shown to contain plasmid DNA (Frazer and Curtiss 1975; Insulburg 1970; Kass and Yarmolinsky 1970). The are able to incorporate labeled precursors into DNA, RNA, and protein (Frazer and Curtiss 1975; Insulburg 1970; Kass and Yarmolinsky 1970; Roozen, Fenwick, and Curtiss 1971; Kool, van Zeben, and Nijkamp 1974; Frazer and Curtiss 1972). Plasmid containing minicells have become a model system for the study of general processes of DNA replication and repair, as well as specific transcriptional and translational products of the plasmid. One of the most common, and most valuable uses of the minicell system, is study of the products of

chimeric plasmids which have been made in vitro recombinant DNA techniques (Frazer and Curtiss 1975; Meagher et al. 1977). This allows detection of plasmid coded products which might be obscured by the high background of RNA and protein synthesis in viable cells. This thesis describes a study of the protein products of recombinant plasmids which carry the E. coli lexA gene.

Genetics of Normal Cell Division and Minicell Production of E. coli

In the normal cell division cycle of E. coli, cell growth, DNA replication, and cell division are closely coordinated. Following the completion of a round of DNA replication and segregation of the nuclear bodies, a septum forms between them leading to the separation of two daughter cells of equal length and DNA content. Completion of a round of DNA replication and synthesis of certain "division proteins" are required for initiation of cell division (for review see Slater and Schaechter 1974).

The complexity of the cell division process is apparent from the large number of genes which participate in its regulation. Cell division mutants fall into several classes. The largest class is composed of temperature sensitive mutants which fail to divide at non-permissive temperature, but which have no other detectable defect (Allen et al. 1972; Ricard and Hirota 1973). Mutants which are thermosensitive for DNA synthesis fail to divide under restrictive conditions due to the direct requirement of DNA synthesis for cell division (Schuster et al. 1973; Hirota, Mordoh, and Jacob 1970; Shapiro et al. 1970). There are several mutants which exhibit abnormal regulation of cell division as

one of several pleiotrophic effects of the mutation. These include lon⁻ mutants, which fail to recover from temporary inhibition of cell division due to low doses of ultraviolet irradiation (Walker and Smith 1970); the tif⁻ mutation in the recA gene (Castellazzi, George, and Buttin 1972), and the tsl⁻ mutation in the lexA gene (Mount, Walker, and Kosel 1973) which cause filamentation at high temperature, and recA⁻ and lexA⁻ mutations which uncouple cell division from DNA synthesis, allowing cell division to occur in the absence of DNA replication (Inouye 1971; Satta and Pardee 1978; Howe and Mount 1975). Mutations in two loci, sfiA and sfiB, have been found to suppress the abnormal filamentation in tif⁻ and lon⁻ mutants (George, Castellazzi, and Buttin 1975).

Minicell producing mutants represent a novel type of cell division aberration in which the fundamental regulation of cell division and the division event itself is normal, but the site of septum formation is abnormal. Minicell producing mutants have been isolated from a number of different genera. In most cases the discovery was accidental since there is no direct selection for minicell producing strains (Frazer and Curtiss 1975). One possible means for selection in the future is the apparent relationship between minicell production and increased resistance to ionizing radiation in E. coli and salmonella strains (Frazer and Curtiss 1975).

The E. coli minicell producing mutant P678-54 was discovered among mutants which had been selected for increased x-ray resistance, but unchanged UV-sensitivity relative to the parental strain P678 (Alder, Fisher, and Stapleton 1966; Adler et al. 1966). Genetic analysis of P678-54 and some of its derivatives has shown that mutations at

two loci are required for production of minicells in E. coli (Frazer and Curtiss 1975; Adler et al. 1966). These are minA, located at 10 minutes, and minB which is located at approximately 29 minutes on the revised linkage map of E. coli (Bachmann, Low, and Taylor 1976). The function of these genes and the nature of the lesions which lead to minicell production are not clear at this time. The x-ray resistance of the minicell producing mutants is determined by two mutations which are transferred with minA and minB respectively, during conjugation (Frazer and Curtiss 1975). Both of these mutations must be present for the increased x-ray resistance.

Properties of Plasmid Containing Minicells

Purified minicells are unable to incorporate radioactive precursors into DNA, RNA, or protein (Cohen et al. 1968b). This is due to low levels of DNA-dependent RNA-polymerase in addition to lack of DNA. Cohen et al. (1968a) found that single stranded DNA transferred to minicells during conjugation with an F^+ or F' donor serves as a template for the synthesis of a complimentary strand. Despite the presence of DNA in conjugated minicells, no RNA or protein synthesis could be detected (Fralick, Fisher, and Adler 1969). Extracts of DNA deficient minicells were shown to support the incorporation of ^{14}C-phenylalanine into acid insoluble material when exogenous polyuridylic acid is supplied (Fralick et al. 1969). These findings suggested that minicells containing endogenous DNA and DNA-dependent RNA-polymerase would be capable of RNA and protein synthesis in vivo.

Minicells purified from plasmid-bearing strains contain plasmid DNA (Insulburg 1970; Kass and Yarmolinsky 1970) and are capable of carrying out DNA, RNA, and protein synthesis (Insulburg 1970; Kass and Yarmolinsky 1970; Roozen et al. 1971; Kool et al. 1974; Frazer and Curtiss 1972). The amount of DNA present and the levels of RNA and protein synthesis varies considerably depending on the particular plasmid contained, growth conditions prior to separation of cells and minicells, and the method used for minicell purification (Insulburg 1970; Roozen et al. 1971).

General properties of plasmid-containing minicells are outlined below.

1. DNA content of the minicell fraction of a culture ranges from .1% to 5% of the total DNA. This fraction is enriched for covalently closed circular DNA, and a large proportion of the DNA is membrane associated (Frazer and Curtiss 1975).
2. Plasmid-containing minicells are capable of DNA synthesis (Frazer and Curtiss 1975; Insulburg 1970; Insulburg 1971). Semiconservative replication of plasmid DNA in minicells has been demonstrated for several different plasmids; the extent of synthesis is estimated to be two rounds of replication of ColE1 DNA, one round for ColV or R222 DNA and less than one round of λdv DNA replication (Frazer and Curtiss 1975; Insulburg 1970; Insulburg 1971).
3. Radiolabeled uridine is incorporated into acid insoluble material at approximately 1% the rate of incorporation by viable cells (Frazer and Curtiss 1975; Roozen et al. 1971). Examination of

the products by electrophoresis and sedimentation velocity centrifugation has shown synthesis of distinct RNA species which are characteristic of the plasmid contained in the minicells (Frazer and Curtiss 1975; Roozen et al. 1971; Kool et al. 1974).

4. Protein synthesis is carried out by plasmid-containing minicells. Total incorporation of radioactive precursors is approximately 1% that of viable cells (Frazer and Curtiss 1975; Roozen et al. 1971). Distinct protein species which are characterized of the plasmid are synthesized (Frazer and Curtiss 1975; Roozen et al. 1971; Kool et al. 1974; Meagher et al. 1977).
5. RNA and protein products synthesized in minicells appear to be functional. Minicells containing conjugal plasmids are competent donors in conjugation experiments (Frazer and Curtiss 1975). Enzyme activity can be assayed in minicells containing plasmids carrying the genes for β-galactosidase (Frazer 1979) or anthrynylate synthetase (Frazer and Curtiss 1973). Regulation of the expression of genes carried on plasmids in minicells appears to be normal in some cases (Frazer and Curtiss 1973).

Due to the low level of macromolecular synthesis in minicells relative to viable cells, minicell preparations which contain less than one viable cell in 10^5 minicells are required for most studies. Minicells can be purified by several cycles of differential centrifugation, filtration, or sedimentation through sucrose or glycerol density gradients.

Biological Role of the lexA Gene Product

The lexA product plays a major role in the response of E. coli to conditions which lead to the arrest of DNA synthesis. This response is characterized by the coordinated expression of a group of inducible functions which include induced mutagenesis and DNA repair, prophage induction, and inhibition of cell division. These events are regulated by the products of the recA and lexA genes. Cells which carry either lexA$^-$ or recA$^-$ mutations are unable to respond to inducing conditions. They exhibit abnormal regulation of cell division and extreme sensitivity to DNA damaging agents such as ultraviolet irradiation (see Witkin 1976 for review). The recA gene is also involved in homologous recombination, but this function appears to be independent of its role in the process of induction.

The mechanism by which the lexA and recA gene products regulate the expression of inducible functions has been largely elucidated. A wealth of genetic data gathered over a considerable period of time have led to the formulation of a variety of models which have then been tested using biochemical techniques. Most of the biochemical investigations which have been carried out have been based on observations about the biological properties of cells which carry mutations in the recA or lexA genes. The major classes of recA and lexA mutations and brief descriptions of their properties are listed in Table 1.

The lexA$^-$ mutation which has been most extensively characterized is the lexA3 allele. Amber mutations in the lexA3 gene have been isolated and found to confer a spr$^-$ phenotype to the cell. The lexA3 mutation is still present, since suppression of the amber mutation

Table 1. Major Classes of Mutations in the recA and lexA Genes.

Mutation	Locus	Type of Mutation	Effect on Induction	Comments and Conclusions	References
recA$^-$	recA	missense and amber	non-inducible	inactivates the recA protein	Mount 1971; Clark and Margulies 1965
tif$^-$	recA	missense temperature effect	thermoinducible	alters the recA protein; tif protein is more active, lethal at high temperature unless accompanied by a sfi$^-$ mutation	George et al. 1975; Gudas and Mount 1977; Castellazzi et al. 1972
lexA$^-$	lexA	missense	non-inducible suppresses tif$^-$ induction	alters the lexA product; lexA protein is more active or is refractory to inactivation	Moody, Low, and Mount 1973
spr$^-$	lexA	missense and amber	suppresses lexA$^-$ inhibition of induction	inactivated lexA protein originally isolated in strain carrying sifA, never recovered without sfi$^-$ mutation	Pacelli, Edmiston, and Mount 1979; Mount 1977
ts1$^-$	lexA	missense temperature effect	suppresses lexA$^-$ inhibition of induction	temperature sensitive lexA protein filaments at high temperature	Mount et al. 1973

restored the lexA3 phenotype. Three independent amber mutations have been isolated, and they are designated lexA3-spr54, lexA3-spr55, and lexA3-spr56 (Pacelli et al. 1979).

The primary biochemical marker for the expression of inducible functions is the rate of synthesis of the recA protein. The recA protein can be seen as a band corresponding to a protein with MW 40,000 when E. coli proteins are displayed on an SDS polyacrylamide gel (Mount 1971; McEntee, Hesse, and Epstein 1976; Little and Kleid 1977; Emmerson and West 1977). It is normally made in small amounts, but its rate of synthesis increases dramatically in response to inhibition of DNA synthesis (Gudas and Pardee 1975; Inouye and Pardee 1970). The amount of recA mRNA has been shown to increase approximately 15 fold under inducing conditions, indicating that the regulation is transcriptional (McPartland, Green, and Echols 1978).

The high level expression of the recA gene appears to be the central event in the biological response to inducing conditions. The recA protein has been purified to apparent homogeneity (Roberts et al. 1978). Activities of the purified protein include high affinity binding to single stranded DNA (Roberts et al. 1978; Gudas and Pardee 1975), DNA-dependent ATPase activity and proteolytic activity. The induction of integrated prophage in response to DNA damage is due to specific cleavage of the lambda repressor of the recA protease (Roberts et al. 1978; Roberts, Roberts, and Mount 1977). It seems likely that most, if not all, of the changes which occur in response to DNA damage result from the activity of the recA protease.

The function of the lexA product appears to be regulation of the level of recA protein synthesis. The pattern of recA protein synthesis in various lexA mutants indicates that the lexA protein is a negative regulator of recA expression; inactivation of the lexA protein is by itself sufficient to cause constitutive high level expression of the recA gene. The lexA3 mutation prevents induction of recA mRNA (McPartland et al. 1978) or protein synthesis (Gudas and Mount 1977; Gudas 1976), while constitutive high level synthesis results from a spr$^-$ mutation (Gudas and Mount 1977).

The high level synthesis of recA protein in spr$^-$ mutants does not cause constitutive expression of inducible functions (Mount 1977), suggesting that some modification of the recA protein occurs in response to inducing conditions. The tif$^-$ mutation in the recA gene causes production of an altered recA protein which becomes active at high temperature (Castellazzi et al. 1972; Gudas and Mount 1977). While the tif$^-$ mutation alone causes constitutive expression of inducible functions at high temperature, the combination of spr$^-$ with the tif$^-$ causes constitutive expression at all temperatures (Mount 1977). The tif$^-$ protein has been shown to be more active than recA$^+$ protein in vitro as well (Roberts et al. 1978).

All of these genetic and biochemical data are consistent with the model for interaction of the lexA and recA gene products in the regulation of induction proposed by Gudas and Mount (1977). The essential points of the model are as follow.

1. The *lexA* gene codes for a repressor of the *recA* gene.
2. Inhibition of DNA synthesis causes modification of the *recA* protein which makes it active as a protease.
3. The *lexA*$^+$ repressor is inactivated under inducing conditions leading to induction of *recA* protein synthesis. The mechanism suggested is proteolytic cleavage by the *recA* protease.
4. *LexA*$^-$ mutants are non-inducible because the *lexA*$^-$ repressor cannot be inactivated by the *recA* protease.
5. Large amounts of *recA* are synthesized and activated, leading to the expression of other inducible functions.

Recent studies in this laboratory have confirmed parts of this model. The product of the *lexA3* gene has been identified as a protein with an approximate MW of 24,000 (Little and Harper, in press). The *lexA*$^+$ protein appears to be a 22Kd protein, although this cannot be conclusively demonstrated because amber mutations in the *lexA*$^+$ gene have not yet been isolated. In vitro cleavage of the putative *lexA*$^+$ protein by purified *tif*$^-$ protein has been demonstrated. The cleavage has the same requirements for ATP and single stranded DNA as the lambda repressor cleavage reaction (Mount 1979). As the model predicts, the *lexA3* product was not cleaved during incubation under identical conditions. Further studies to examine the possibility that the *lexA* protein functions as a repressor of the *recA* gene would be aided by the availability of a source of *lexA* protein which could be used for in vitro assays of its activity. Toward this end, Little (in press) has constructed a series of plasmids which carry the *lexA*$^+$, *lexA3*, and amber *spr* alleles. The phenotypes of cells containing the *lexA3* or *lexA*$^+$ plasmids indicate that the *lexA*

products are functional and over-produced (Mount, Little, and Edmiston, in press). This thesis presents a study of the products synthesized by minicells containing these plasmids, and an initial investigation of the regulation of *lexA* gene expression.

CHAPTER 2

MATERIALS AND METHODS

Bacterial and Plasmid Strains

The minicell producing mutant used in this study is DS410 which was obtained from Reeve (1977). It is described as a prototrophic Su^- derivative of stain X925 (Reeve 1977) which is a single colony isolate of the original minicell producing mutant P678-54 (Frazer and Curtiss 1975). Plasmid strains used are shown in Table 2.

Nitrous Acid Mutagenesis of pJL21 DNA

pJ121 DNA was diluted 40 times into 0.1M sodium acetate (NaOAc) pH 4.0. A sample was taken and diluted 4 fold into 0.1M Tris-HCl (pH 8.0). $NaNO_2$ was added to the reaction tube to a final concentration of 0.1M. Samples were withdrawn and diluted into Tris-HCl at times ranging from 0.5 minutes of treatment to 20 minutes. This DNA was used to transform the minicell producing strain, transformants were selected for tetracycline resistance and were screened for altered UV-sensitivity by a patch test.

Preparation of Competent Cells and Transformation with Plasmid DNA

Competent cells were prepared by a modification of the method of Cosloy and Oishi (1973). Cells were grown to approximately 5 x 10^8 cells/ml in L-broth, chilled, and pelleted. They were washed once in cold .85% saline, then suspended in an equal volume of cold 30 mM $CaCl_2$,

Table 2. Plasmid Strains.

Plasmid	Selective Marker	Effect on UV* Sensitivity	*lexA* allele	Reference
pBR322	Tc^R Ap^R	no effect	none	Bolivar et al. 1977
pJL21	Tc^R	+	*lexA*$^+$	Little, in press
pJL26	Tc^R	++++	*lexA3*	
pJL30	Tc^R	±	*lexA3-spr54*	
pJL31	Tc^R	±	*lexA3-spr55*	
pJL32	Tc^R	±	*lexA3-spr56*	
pLX1	Tc^R	++	(*lexA*$^+$)	Derived from pJL21 by nitrous acid mutagenesis
pLX2	Tc^R	++	(*lexA*$^+$)	
pLX3	Tc^R	++	(*lexA*$^+$)	
pLX4	Tc^R	++	(*lexA*$^+$)	
pLX5	Tc^R	++	(*lexA*$^+$)	
pSP1	Tc^R	±	(*lexA*$^+$-*spr*)	Derived from pJL21 by nitrous acid mutagenesis
pSP2	Tc^R	±	(*lexA*$^+$-*spr*)	

*moderate increase on sensitivity +
significant increase on sensitivity ++
extreme sensitivity ++++
slight effect on sensitivity ±

and left on ice for 20 minutes. The cells were pelleted and suspended in one-tenth volume 30 mM $CaCl_2$-15% glycerol. Two hundred microliters of the competent cells were added to a tube which contained 0.05 ml of mutagenized pJL21 DNA and 0.05 ml of 0.1M $CaCl_2$. This was incubated at 0° for 1 hour, then heat shocked at 42° for 2 minutes and chilled in an ice water bath. Four mls of L-broth were added to each tube and the cells were incubated at 37° for 2 hours. Tryptone plates containing 20 µg/ml of tetracycline were used for selection of transformants.

Purification of Minicells

Purification of minicells was essentially as described by Frazer and Curtiss (1975). Minicell producing strains were grown to late log phase in L-broth, or M9 minimal medium supplemented as described in this chapter under Media and Solutions. The cells were collected by centrifugation at 10,000 x g for 20 minutes, and suspended in BSG at approximately 10 to 50 times their original concentration. The cell suspension was vortexed vigorously for 2 minutes and layered on a 5 to 20% linear sucrose gradient. The gradients were centrifuged at 1000 x g for 25 minutes. The minicells form a band approximately 1/4 of the way down the tube. This band was removed from the top of the tube using a syringe with the needle bent at a right angle. Minicells were slowly diluted with an equal volume of BSG, pelleted, and resuspended in a volume of BSG suitable for layering on a second gradient. The entire process of sucrose gradient centrifugation was repeated, and the minicells removed from the second gradient were pelleted and suspended in 1 ml of BSG. The number of minicells was determined using a

Petroff-Hausser counter, and an aliquot was diluted and plated to determine the concentration of viable cells. Minicell preparations purified by this method should contain fewer than one viable cell for every 10^6 minicells.

^{35}S-Methionine Labeling of Proteins Synthesized in Minicells

Minicells were suspended in ^{35}S-methionine labeling medium at a concentration of 2 x 10^{10} per ml. They were incubated at 37° for 30 minutes, then ^{35}S-methionine (specific activity at least 500 Ci/µmole) was added to give a final concentration of 20 µCi/ml. After 20 minutes at 37°, labeling was stopped by addition of unlabeled methionine to a final concentration of 200 µg/ml and chilling the samples to 0°. The labeled minicells were washed twice with 10 mM Tris-HCl pH 7.4, suspended in a small volume of 10 mM Tris-HCl containing 20% glycerol, and frozen.

^{14}C-Amino Acid Labeling of Proteins

Purified minicells were suspended in ^{14}C-amino acid labeling medium at a concentration of 2 x 10^{10}. They were preincubated for 30 minutes, then labeled with 20 µCi/ml neutralized ^{14}C-amino acid mixture for 20 minutes at 37°. Labeling was stopped by addition of casamino acids to a final concentration of .83% and chilling the samples. The labeled minicells were processed as described for ^{35}S-methionine labeled minicells.

SDS Polyacrylamide Gel Electrophoresis

Electrophoresis was performed as previously described (Little and Harper, in press). All gels were 15% polyacrylamide final concentration. Fluorography was done according to the method of Bonner and Laskey (1974).

Incorporation of ^{35}S-Methionine into Acid Insoluble Material

Minicells were purified from DS410 and DS410(pBR322) as described above. They were suspended in ^{35}S-methionine labeling medium at a final concentration of 2 x 10^{10} minicells/ml. Two aliquots of each strain were incubated at 37°. ^{35}S-methionine (20 µCi/ml) was added at 0 minutes to one aliquot of each, and at 30 minutes to the other aliquot. Fifty µl samples were taken at 0, 5, 10, 20, 30, 40, and 50 minutes after addition of the label. They were spotted on Wattman 3MM filters which had been previously soaked in 10% TCA and dried. At the end of the experiment, all filters were allowed to dry, then they were washed twice in cold 10% TCA for 30 minutes, washed once in cold 95% ethanol, dried, and counted.

Incorporation of ^{35}S-Methionine into Polypeptides Synthesized in Minicells Containing pBR322

Minicells were purified from DS410(pBR322) as described above. They were suspended in ^{35}S-methionine labeling medium at a concentration of 2 x 10^{10}/ml. ^{35}S-methionine (20 µCi/ml) was added and 100 µl samples were taken at 0, 2, 5, 10, 15, and 20 minutes. Labeling was stopped by adding the sample to 1 ml frozen M9 medium containing 200 µg/ml cold methionine. Samples were processed as described above,

and polypeptides synthesized were examined by SDS polyacrylamide gel electrophoresis. An autoradiograph of the gel was scanned using a densitometer.

Media and Solutions

Cells were grown in L-broth or M9 minimal medium supplemented with 0.5% glucose, 1.0% casamino acids 1mM $MgSO_4$ and 0.2 mM $CaCl_2$ as described by Frazer and Curtiss (1975). Strains carrying one of the Tc^r plasmids were grown in the presence of 10 μg/ml.

^{35}S-Methionine Labeling Medium

^{35}S-methionine labeling medium was M9 minimal medium supplemented with 50 μg/ml of each of the unlabeled amino acids, 50 μg/ml uracil, 50 μg/ml adenine, and either 3% glycerol or .5% glucose.

^{14}C-Amino Acids Labeling Medium

^{14}C-amino acids labeling medium contined 50 μg/ml each cysteine, glutamine, asparagine, methionine, and tryptophan; 50 μg/ml uracil, 50 μg/ml adenine and either 3% glycerol or 0.5% glucose. Immediately before the labeling, freshly made cycloserine solution was added to a final concentration of 20 μg/ml.

Neutralization of the ^{14}C-Amino Acid Mixture

Neutralization of the ^{14}C-amino acid mixture (New England Nuclear, 01. mCi/ml in .1N HCl) was necessary because a large volume of the mixture had to be added in order to label the minicells efficiently. This was done by adding one-tenth volume of 10X M9 salts to buffer the solution, then adding one-tenth volume 1M NaOH.

Sucrose Gradients

Sucrose gradients used for the purification were buffered in BSG (1) which is phosphate buffered saline containing .85% NaCl, .3% KH_2PO_4, .6% Na_2HPO_4, and .1 mg/ml gelatine. This buffer was made up 10X and diluted. Standard sucrose solutions used were 5% and 10% sucrose in BSG. Sucrose solutions were sterilized by autoclaving.

CHAPTER 3

RESULTS

Incorporation of ^{35}S-Methionine by DNA Deficient and Plasmid-Containing Minicells

Incorporation of ^{35}S-methionine into trichloroacetic acid insoluble material by minicells purified from DS410 and DS140(pBR322) is shown in Figure 1. Plasmid containing minicells incorporate approximately three times as much label as DNA-deficient minicells. Preincubation for 30 minutes prior to the addition of label leads to greatly increased incorporation by plasmid-containing minicells with only a slight increase in incorporation by DNA-deficient minicells. This result is consistent with previous reports (Frazer and Curtiss 1975; Meagher et al. 1977). Incubation of cells in sucrose solutions causes reduced protein synthetic capability. Since the purification procedure involves sucrose gradient centrifugation, the minicells are probably effected the same way. Recovery from the sucrose effects during the preincubation would account for the increased incorporation of label.

Similar kinetics were observed when incorporation of ^{35}S-methionine into the β-lactamase coded for by the ampicillin resistance gene of pBR322 was examined. Minicells purified from DS410(pBR322) were preincubated and labeled as described in Chapter 2, Materials and Methods. Samples were taken at various times after addition of the label and analysed by SDS polyacrylamide gel electrophoresis. An autoradiograph of the gel was scanned with a densitometer, and the height of the

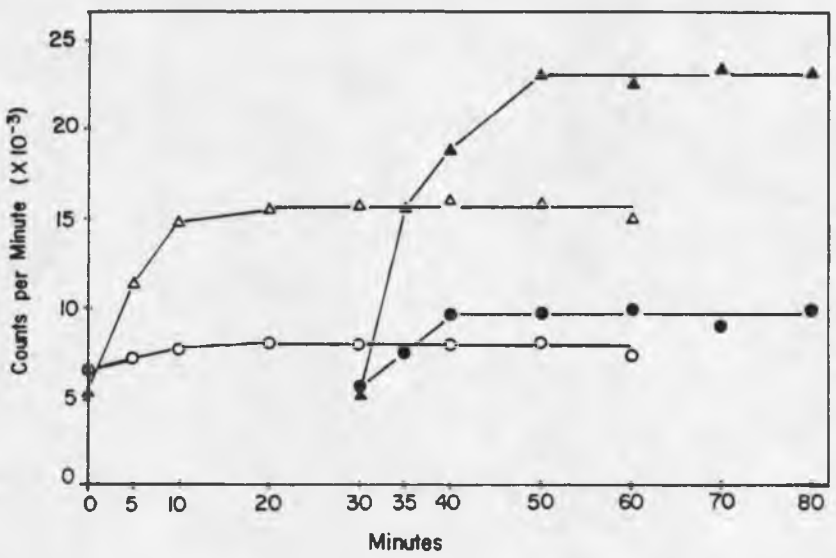

Figure 1. Incorporation of ^{35}S-Methionine in Trichloroacetic Acid Insoluble Material by Minicells Purified from DS410 and DS410(pBR322) -- Minicells purified from DS410 (circles) and DS410(pBR322) (triangles) were suspended in ^{35}S-methionine labeling medium. Two aliquots from each strain were incubated at 37°. Label was added to one aliquot of each at 0 minutes (open symbols) and to the other aliquot at 30 minutes (closed symbols). Acid insoluble CPM in 50 µl was determined for samples taken at 0, 5, 10, 20, 30, 40, and 50 minutes after addition of label.

peak corresponding to the β-lactamase band was measured. Figure 2 shows the result of this experiment.

Products of the lexA3 and Amber spr Plasmids

The fluorograph in Figure 3 shows the polypeptides synthesized in minicells containing the plasmids pBR322 (lane 2), pJL26 which carries the lexA3 gene (lane 3), and plasmids carrying the three different amber spr mutations (lanes 4 through 6), as well as DNA-deficient minicells (lane 1). The absence of bands in lane 1 demonstrates that the label incorporation by DNA-deficient minicells as seen in Figure 1 does not represent synthesis of stable polypeptides.

Four major polypeptides are synthesized by minicells containing pBR322 (lane 2). The two extremely heavy bands represent the β-lactamase and its unprocessed precursor. The molecular weights of these proteins are approximately 28,000 and 29,000 respectively. The other two bands represent the 34Kd protein which is responsible for tetracycline resistance, and a 25Kd protein of unknown function.

Purified minicells containing the lexA3 plasmid (lane 3) do not synthesize the β-lactamase bands because the DNA fragment which carries most of the β-lactamase gene was removed from pBR322 and replaced by the bacterial fragment carrying the lexA3 gene during construction of this plasmid (Little, in press). The 25Kd protein is not synthesized in these minicells, indicating that some part of the coding and regulatory sequences required for its synthesis are carried on the fragment of pBR322 DNA which was removed. It is possible that the 25Kd protein is related to the β-lactamase. pJ126 directs the synthesis of the

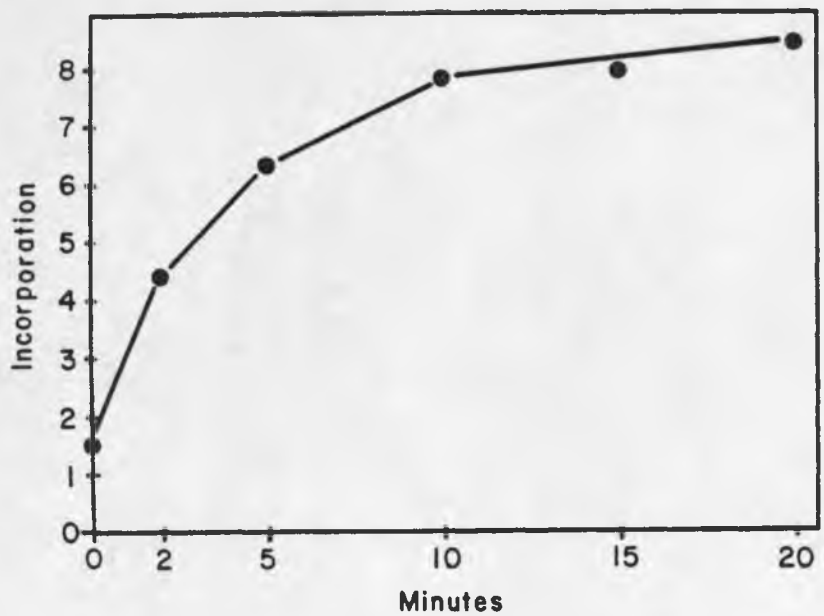

Figure 2. Incorporation of ^{35}S-Methionine into the Plasmid Coded β-lactamase in Minicells Containing pBR322 -- The polypeptides labeled in minicells containing pBR322 were analysed by SDS polyacrylamide gel electrophoresis. An autoradiograph of the gel was scanned by a densitometer, and the height of the peak corresponding to the β-lactamase band was measured. The relative amount of incorporation into the β-lactamase is given in arbitrary units.

34Kd tetracycline resistance protein, and a new protein which migrates very close to the tet protein, as well as the 24Kd lexA3 protein.

The plasmids pJL30, pJL31, and pJL32 are derivatives of pJL26 which carry the amber spr mutations lexA3-spr54, lexA3-spr55, and lexA3-spr56 respectively. Comparison of the products of the amber plasmids (lanes 4 through 6) with those of the lexA3 plasmid reveals two major differences: the lexA3 protein is not synthesized in minicells containing the amber spr plasmids, and a new protein is synthesized in each strain. The new bands appear to represent amber fragments of the lexA3 protein. Although direct analysis such as partial peptide mapping or amino acid sequencing would be necessary to prove this relationship, the circumstantial evidence to support this supposition is good. The size of the new protein synthesized is different for each plasmid, and these bands represent the only new products of the amber spr plasmids which can be detected. The density of the bands corresponding to the lexA3-spr54 and lexA3-spr56 fragments (lanes 4 and 6) indicate that they are synthesized at 20 to 30 times the rate of lexA3 protein synthesis. The lexA3-spr55 fragment (lane 5) appears to be extensively degraded; however, the band is still 4 times as intense as the lexA3 band. These results indicate that the low level of lexA protein synthesis observed for the lexA3 and putative $lexA^+$ products is due to some form of negative regulation rather than an inherent inability of the gene to be expressed at a high rate. In addition to this information, the high level expression of the amber fragments indicates that the lexA product participates in this negative regulation.

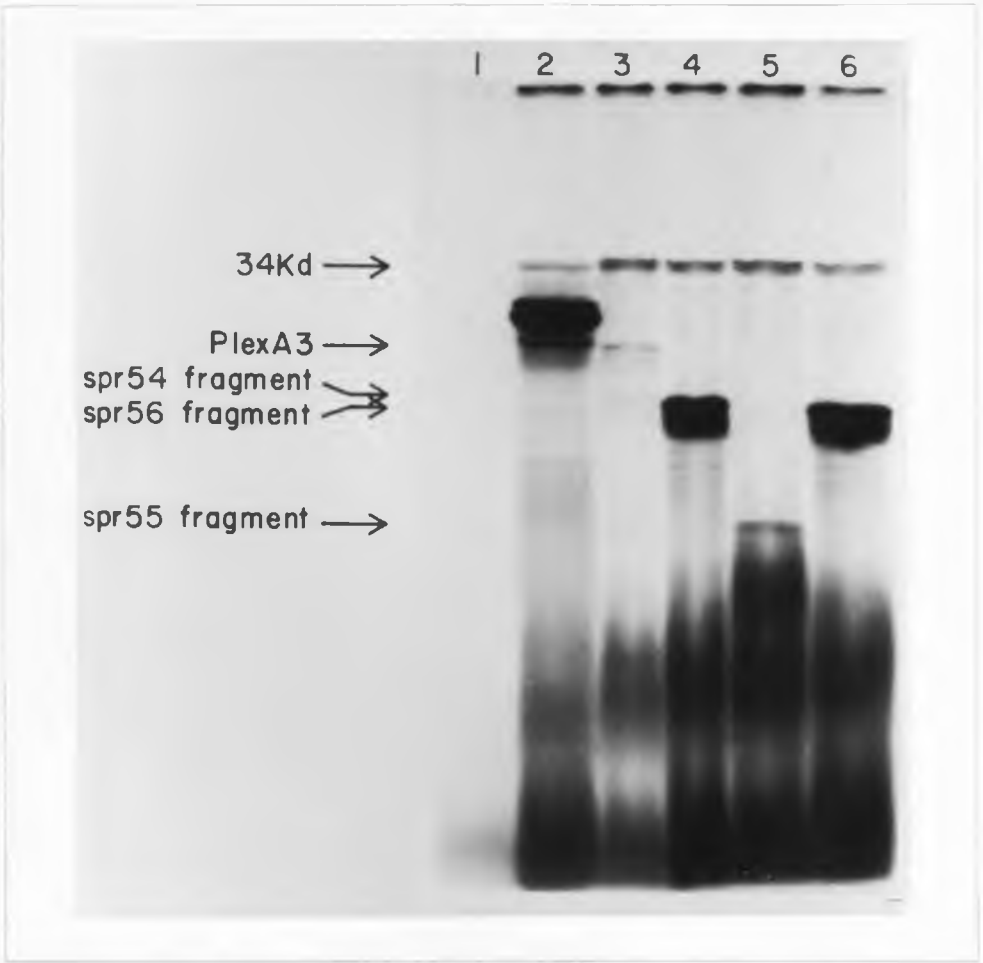

Figure 3. ^{14}C-Amino Acids Labeled Products of Minicells Containing Various Plasmids -- Products of minicells containing (1) no plasmid, (2) pBR322, (3) pJL26, (4) pJL30, (5) pJL31, and (6) pJL32.

Isolation and Characterization of a Mutant lexA Plasmid

The over-production of the lexA amber fragments indicates self-regulation of the lexA gene. If this regulation is direct, then mutations which lead to the constitutive high level expression of the lexA gene should fall into two general classes: (1) those which alter the lexA product so that it no longer functions as a regulator, and (2) those which alter a regulatory site in the gene so that its expression is insensitive to regulation. Mutations in genes other than lexA could alter its regulation if the function of the lexA protein in regulation is indirect. Isolation of regulation defective mutations in the lexA gene carried on a plasmid offers the advantage of limiting the possible mutations to the lexA gene or very closely linked sites, in addition to the obvious advantage of being able to screen for over-production in minicells.

The screen for mutants with altered regulation of the lexA gene was based on the properties of cells carrying lexA plasmids. The presence of the $lexA^+$ plasmid, pJL21, has been shown to cause moderate sensitivity to UV-irradiation in several different hosts (Mount et al., in press). This effect may be due to over-production of the lexA protein as a result of the numerous copies of the gene in these cells. The presence of an amber spr plasmid causes only a slight increase in UV-sensitivity in a non-suppressor host. It was expected that a regulatory site mutation which allowed high levels synthesis of functional lexA protein would increase the UV-sensitivity of the host considerably, while inactivation of the lexA product would lead to decreased

UV-sensitivity as is seen for the amber spr plasmid. PJL21 DNA was mutagenized with nitrous acid in vitro and used to transform the minicell producing strain DS410. In order to assure the isolation of independent mutants, a series of separate transformations were done; and only one mutant plasmid from each was finally selected. Tetracycline resistant transformants were screened for altered UV-sensitivity relative to DS410(pJL21). A number of transformants with slightly increased UV-sensitivity and two with reduced sensitivity were isolated. Five mutant plasmids which cause increased UV-sensitivity were designated pLX1 through pLX5, and the plasmids carrying new spr mutations are pSP1 and pSP2.

The proteins synthesized in minicells containing these plasmids were examined by SDS polyacrylamide gel electrophoresis. The fluorograph in Figure 4 shows the proteins labeled in minicells containing pJL26 (lane 1) and the mutant plasmids pLX1, pLX2, pSP1, pLX3, pLX4, pSP2, and pLX5 (lanes 2 through 8 respectively). All of the mutant plasmids code for the 34 Kd proteins and the protein believed to be the $lexA^+$ product. The amount of label in each band was quantitated by microdensitometer tracing of the fluorograph. The ratio of lexA protein/34Kd band was calculated. This value is normally 0.3 for products of the lexA3 plasmid and 0.15 for pJL21. The ratio for the products of mutant plasmids is approximately 0.15 for all except pSP2. The average lexA/34Kd ratio for the products of pSP2 is 1.5 calculated from tracings of fluorographs of several differents gels. This increase

Figure 4. Proteins Labeled in Minicells Containing pJL26 and the Mutagenized <u>lexA</u>$^{+}$ Plasmids -- Products of minicells containing (1) pJL26, (2) pLX1, (3) pLX2, (4) pSP1, (5) pLX3, (6) pLX4, (7) pSP2, and (8) pLX5.

in the relative amount of *lexA*$^{+}$ protein synthesized in minicells containing pSP2 can be seen in the densitometer tracings shown in Figure 5.

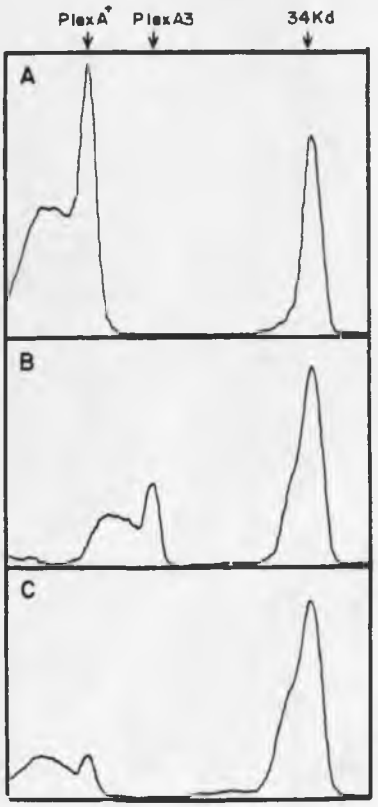

Figure 5. Densitometer Tracings of Lanes 1, 2, and 7 of the Fluorograph Shown in Figure 4 -- Densitometer tracings were done to determine the relative amount of lexA protein synthesis directed by pJL26 and the mutant plasmids. The height of the 34Kd peak was adjusted so that it remained constant for all lanes. Three tracings shown represent the products specified by (A) pSP2, (B) pJL26, and (C) pLX1. The profile of pLX1 is typical of those seen for the other mutant plasmids and for pJL2.

CHAPTER 4

DISCUSSION

Autogenous regulation is defined as "the mechanism by which a protein directly controls expression of its own structural gene (Goldberger 1974, p. 810)." Conclusive proof of autogenous regulation requires demonstration that the protein influences the level of transcription of its structural gene, or translation of its mRNA in vitro. The observation that a mutation which alters the function of a protein also alters its rate of synthesis suggests autogenous regulation, but this interpretation is not always correct. The recA gene is an excellent example of a gene which is subject to a self-regulation similar to autogenous regulation, but which is not autogenous in the strict sense of the term.

The recA protein influences the expression of its own gene indirectly, presumably by inactivation of the $lexA^+$ protein. Truly autogenous positive regulation of the recA gene would require that the recA protein directly promote transcription of the recA gene. While the distinction between autogenous regulation and indirect self-regulation may seem subtle, it can be an important consideration in a discussion of regulatory pathways. The distinguishing feature of true autogenous regulation is that mutational alteration of the regulation of a gene is possible only by a mutation in the gene itself. This could be advantageous in the case of a regulatory protein which must respond

rapidly to changes in the intracellular environment, but which would be deleterious to the cell if it were synthesized at a high rate.

Regulation of lexA Gene Expression

The results of this study indicate that the lexA product participates in the negative regulation of the lexA gene. This interpretation is based on the correlation between inactivation of the lexA protein and an increased rate of synthesis of proteins which are believed to be the products of the mutant lexA genes.

The polypeptides synthesized in minicells containing the amber spr plasmids include a new product specified by each plasmid. Rigorous proof that these polypeptides are amber fragments of the lexA3 protein is not available; several observations suggest that they are amber fragments. The size of the new protein specified by the plasmid is different for each of the three amber spr plasmids. The only difference between the three plasmids should be the particular amber allele present. Specialized transducing phages carrying the spr54 and spr55 alleles direct the synthesis of fragments in a non-suppressor host which are the same size as those coded for by the corresponding plasmid. This observation must be qualified by the fact that the size comparison is approximate because the samples have not been run of the same gel. The synthesis of the allele specific fragment by both the plasmid and the phage carrying a particular amber spr mutation indicates that the fragment is coded for by the inserted bacterial DNA, rather than the result of read-through from the adjacent plasmid sequences. If we accept the arguments supporting the probable identity of these polypeptides as

amber fragments of the lexA3 protein, the results clearly indicate self-regulation of the lexA3 gene.

Additional evidence for self-regulation of the lexA gene is the isolation of the mutant plasmid which carries a new spr mutation and which directs the synthesis of approximately ten times the normal amount of the putative $lexA^+$ protein. The isolation of mutant plasmids was a biological test of the idea that the lexA gene is self-regulating: the mutant was selected on the basis of altered lexA function and was found to have altered regulation of lexA expression. While it is possible that these two properties of pSP2 are due to independent mutations, this is not very likely because pSP2 was isolated from the pJL21 DNA given the shortest time of nitrous acid treatment (0.5 minutes). The plasmids given this dose were able to transduce cells to tetracycline resistance 80%, as well as control plasmids. Therefore, the probability of two mutations in the plasmid DNA is not high.

A general pattern of lexA regulation cannot be deduced from the results of the mutant plasmid isolation experiments due to the small number of mutants characterized. The cause of the increased UV-sensitivity of cells carrying pLX1 through pLX5 is not clear. This could be due to mutations which cause increased copy number of the plasmid; however, it is unlikely that this is responsible in all five cases. The most likely explanation is that these plasmids carry mutations which make the lexA protein slightly more active or slightly less susceptible to inactivation. Further characterization of pSP1, which appears to carry a new spr mutation, but does not direct the over-production of the putative $lexA^+$ protein, could be very interesting.

While it is possible that the reduced UV-sensitivity of cells containing pSP1 is due to a reduced copy number, it is also possible that the plasmid carries a mutation which causes altered *lexA* function but allows normal regulation of *lexA* synthesis. Such a mutation would provide a great deal of information about *lexA* regulation.

In addition to the evidence presented here, self-regulation of *lexA* expression is indicated by the results of experiments using the specialized transducing phage carrying the *lexA* gene. We have found that the presence of the *lexA3* plasmid in the host greatly inhibits synthesis of the *lexA3* protein and the *spr54* amber fragment. The presence of a *lexA*$^+$ plasmid inhibits synthesis approximately two-fold while an amber *spr* plasmid does not have any effect on the amount of synthesis (Little and Harper, in press).

Self-regulation of the *lexA* gene may represent a means by which the cell can quickly return to a normal state after induction. The *recA* protein appears to be modified in response to inducing conditions, possibly causing increased protease activity.[1] The modified *recA* protein is believed to inactivate the *lexA*$^+$ product, leading to high-level synthesis of *recA* protein. If *lexA* is self-regulating, then inactivation of the *lexA*$^+$ protein would also lead to high-level expression of the *lexA* gene. This would allow rapid accumulation of a normal amount of *lexA* protein when the inducing signal is reduced, leading to repression of *recA* protein synthesis.

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1. Refer to page 11, items 1 through 5.

Future Investigations

The results of this study leave several basic questions to be answered. Two of these are:

1. Is *lexA* regulation at the level of transcription or translation?
2. Is *lexA* self-regulation direct or indirect?

The first question might be answered by comparison of the amount of *lexA* specific transcription in minicells containing the $lexA^+$ plasmid and the mutant plasmid, plasmid pSP2.

The second question will be extremely difficult to answer. The *lexA* protein is made in such small amounts that it cannot be seen when total *E. coli* proteins are displayed on a gel.

Investigations, such as this, using minicells containing mutant *lexA* plasmids are limited to studying the effect of mutations in or closely linked to the *lexA* gene. The most effective approach to answering the question of direct regulation would be to determine the effect of *lexA* protein on the transcription and translation of the *lexA* gene in vitro.

NOMENCLATURE

allele — One of several possible forms of a gene.

gene — The basic unit of hereidity. Functional unit which occupies a specific site on the DNA (or RNA) and is capable of transcriptional or translational expression. In most bacterial systems each gene is given a three letter designation (often mnemonic) which is often followed by a capital letter to distinguish between different genes which effect the same process. Different alleles are indicated by their individual allele number, or by the superscript symbol (-) to indicate a mutant allele, or plus (+) to indicate the wild type allele. Names of genes are italicized. Examples: *lexA3*, *lexA*$^{+}$, *recA*$^{-}$.

genotype — The genetic makeup of an organism. Designation is like that for the gene. Examples: *lexA*$^{-}$, *spr*$^{-}$.

locus — Chromosomal location of a gene.

operator — Region on DNA with which a specific repressor interacts to regulate the expression of genes in an operon.

operon — One or more genes in a single transcriptional unit whose expression is regulated by an adjacent operator region and a specific repressor.

phenotype — Physical appearance of an organism resulting from its genotype and environmental factors. Designated by the name of the the gene, capitalized and not italicized. Examples: LexA^{-}, RecA^{+}, Spr^{-}.

promotor — Region on the DNA which is required for initiation of transcription.

repressor — Protein product of a regulatory gene, capable of combining with the operator region on the DNA or mRNA to inhibit expression of the genes in an operon.

wild type — Form most commonly found in nature, or form generally considered to be normal. The term may apply to a particular allele, or to the phenotype of an organism. Designated by the symbol (+).

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