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IDENTIFICATION OF A ØX174 CODED PROTEIN INVOLVED IN THE INHIBITION OF β-GALACTOSIDASE SYNTHESIS IN ESCHERICHIA COLI

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SUMMARY : The synthesis of β-galactosidase (EC 3.2.1.23 : β-D-galactoside galactohydrolase) in Escherichia coli is repressed as a result of infection with single-stranded DNA phage ØX174. An amber mutant in ØX174 cistron A, which codes for two proteins, does not inhibit the enzyme synthesis while amber mutants in all other genes do cause repression. A mutant near the amino-terminal end of cistron A, which produces the small 35,000 molecular weight cistron A polypeptide, also inhibits the synthesis of β-galactosidase. Inhibition is also observed in an Escherichia coli rep mutant which does not support the replication of replicative-form DNA. Exogenous nucleotide bases and cyclic 3',5'-adenosine monophosphate (cyclic AMP) do not have any effect on the degree of repression.

INTRODUCTION : The single-stranded Deoxyribonucleic Acid containing bacteriophage ØX174 has been found to inhibit host macromolecular synthesis, in general, at a multiplicity of infection (moi) exceeding 4 (1). It had been earlier reported (2) that synthesis of the enzyme β-galactosidase was significantly repressed in Escherichia coli C upon infection with ØX174 am 3 at moi 5-7. It was thereafter concluded from a series of experiments that the repression was caused by phage gene-coded product(s).

Current knowledge about the small isometric phage ØX174 indicates that the phage DNA encodes nine genes, which specify at least 16 gene products (3). The genes are designated A through H, and J. Induction of β-galactosidase was carried

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out in cells infected with different mutants of ØX174. It was observed that at least one of the two mutants in cistron A does not inhibit the enzyme synthesis whereas mutants in seven other cistrons (B, D, E, F, G, H and J) do cause inhibition. Cistron A of ØX174 contains an internal initiator and codes for two polypeptides whose molecular weights are approximately 60,000 and 35,000 daltons respectively. Amber mutants near the carboxy-terminal end of the gene do not synthesise any of the two but those near the amino-terminal end, however, synthesise the smaller polypeptide (4). This report shows that an amber mutant in cistron A which produces the small A protein also causes inhibition of the enzyme indicating that this protein may be involved in the inhibition.

Inhibition was observed in an Escherichia coli rep mutant which does not support the replication of replicative-form (RF) DNA of the phage. This indicates that RF DNA replication by itself is not responsible for the observed inhibition. Also the inhibition is not due to depletion in nucleotide pools as addition of nucleotide bases to the media had no effect on the degree of inhibition.

MATERIALS AND METHODS : Bacteria and Bacteriophage : Escherichia coli C (C122) which is the normal host for ØX174 was obtained from Dr. R. L. Sinsheimer. Phage ØX174 mutants am3 (cistron E), am 210 (cistron B), am 10 (cistron D), am 70 (cistron F), am 9 (cistron G), am 23 (cistron H), am 6 (cistron J), am 62 and am 8 (both cistron A) were obtained from Dr. I. Tessman and Dr. G. N. Godson. E. coli CR rep3 (D32) was provided by Dr. David T. Denhardt.

Experimental Procedure : E. coli C was grown in Tris-glycerol (TG) Medium containing per liter : 12 g of tris(hydroxymethyl) aminomethane (Tris); 35 mg KCl; 1 g NH_4Cl; 0.2 g $MgCl_2.6H_2O$; 2.7 mg $FeCl_3.6H_2O$; 68 mg NaCl; 0.4 g KH_2PO_4; 0.3 g Na_2SO_4 and 10 ml glycerol at pH 7.5. Before phage infection, $CaCl_2$ was added to the growth medium to a final concentration of 2×10^{-3} M. Cells were grown to the middle of the log phase (about

2-3 x 10^8 cells per ml), infected at a moi 5-7 and induced for the enzyme β-galactosidase. Induction was done with isopropyl-β-D-thiogalactopyranoside (IPTG) added to a final concentration of 0.11 mg per ml (i.e. 4.6 x 10^{-4} M).

Assay of β-galactosidase was carried according to the method of Pardee and Prestige (5) as modified by Pollard and Davis (6). One unit of β-galactosidase was defined as the amount of enzyme that would produce 1 nmol of o-nitrophenol per minute at 37°C, pH 7.0.

RESULTS : (i) Inhibition of β-galactosidase synthesis in cells infected with different amber mutants of ØX174. Log phase E. coli C cells were infected with different amber mutants of ØX174 and then induced with IPTG. Induction was carried out 15 minutes after phage infection when phage-specific proteins were abundantly present. In cases of mutants other than that in cistron E (lysis defective), lysis was delayed by adding $MgSO_4$ to the growth media just before infection (7). Enzyme levels in infected cells were compared with that in uninfected cells 60 minutes after infection.

Table 1 shows the results obtained with amber mutants in 8 of the 9 ØX174 cistrons. It appears therefrom that repression of β-galactosidase synthesis observed 60 minutes after infection ranges from 65 to 80 per cent for at least 7 mutants. Only in the case of infection with am 62, containing a mutation in cistron A, the repression was not more than 4 per cent. Taking this to be well within the limits of experimental error it may be inferred that am 62 fails to repress the induction of β-galactosidase synthesis in E. coli.

Similarly, when the results obtained with am 62 and am 8, both containing mutations in cistron A, are compared, it transpires that while the former fails to inhibit the induction of β-galactosidase, in the case of the latter inhibition is nearly 85 per cent (Fig. 1).

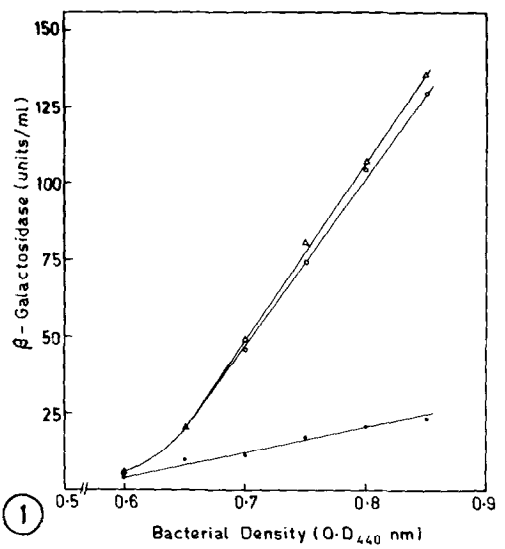
Table 1

Repression of β-galactosidase in Escherichia coli C infected with different amber mutants of ØX174

Phage mutant	Mutation in cistron	Repression observed 60 minutes after infection
am 210	B	65 %
am 10	D	72 %
am 3	E	73 %
am 70	F	78 %
am 9	G	78 %
am 23	H	80 %
am 6	J	79 %
am 62	A	4 %
am 8	A	85 %

E. coli C was grown in TG medium as described in Materials and Methods. One part of the culture was infected with a mutant of ØX174 at 0 min (OD_{440} nm of the culture = 0.55) at a moi = 6; the other part was treated as the uninfected control. In the cases of all mutants except am E 3, $MgSO_4$ was added to the media to a final concentration of 0.2 M just before infection. Inducer IPTG was added to both parts 15 min later. Two ml aliquots were withdrawn at suitable intervals : 1 ml was collected over 0.2 ml of toluene at $0^{\circ}C$ and shaken vigorously; the other half was used for OD determination at 440 nm. Assay of β-galactosidase was carried out as described in Materials and Methods.

(ii) Inhibition of β-galactosidase synthesis in E. coli rep mutants. Since cistron A mutants are unable to replicate phage RF DNA within the cell (8,9) it needed to be ascertained whether the process of replication itself had a bearing on the inhibition of β-galactosidase synthesis. For that the induction experiment was carried out with E. coli CR rep 3, a mutant in which the replication of RF DNA does not occur (10). In this case, too, it was found that the repression of the enzyme



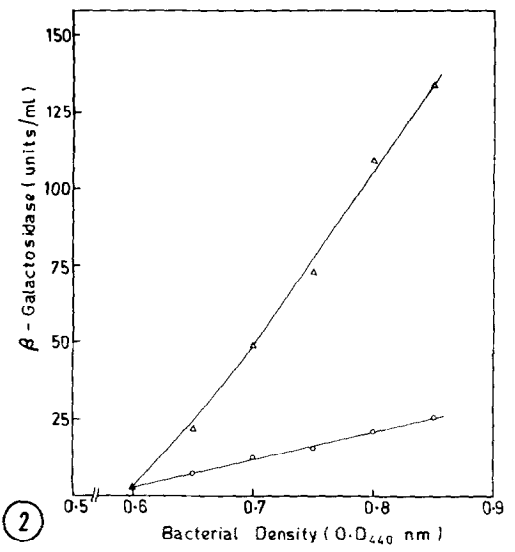


Figure 1. Effect of ϕX174 cistron A mutants on the synthesis of β-galactosidase in E. coli C. E. coli C was grown in TG medium as described in Materials and Method. $MgSO_4$ was added to the medium to a final concentration of 0.2 M. It was divided into three parts. One part was treated as the uninfected control; the other two parts were infected with ØX174 am A 62 and am A 8 respectively both at moi = 6. Inducer IPTG was added to all three parts 15 min later. Aliquots were withdrawn at suitable intervals and assayed for β-galactosidase. △—△—△—△ uninfected control; ○—○—○—○ infected with am A 62; ●—●—●—● infected with am A 8.

Figure 2. Inhibition of β-galactosidase synthesis in E. coli rep mutant. E. coli CR rep 3 (D32) was grown in TG medium supplemented with 10 µg thymine/ml. and divided into two parts - one part being treated as the uninfected control while the other infected with ØX174 am E 3 at moi = 6. IPTG was added 15 min later. Aliquots were withdrawn at suitable intervals and assayed for β-galactosidase. △—△—△—△ uninfected control; ○—○—○—○ infected.

60 minutes after infection was just over 80 per cent (Fig.2). Thus, it became evident that the inhibition phenomenon is not dependent on phage DNA replication. At the same time the possible argument that the phage DNA replication may cause depletion in nucleotide pools leading to repression of enzyme synthesis is also weakened.

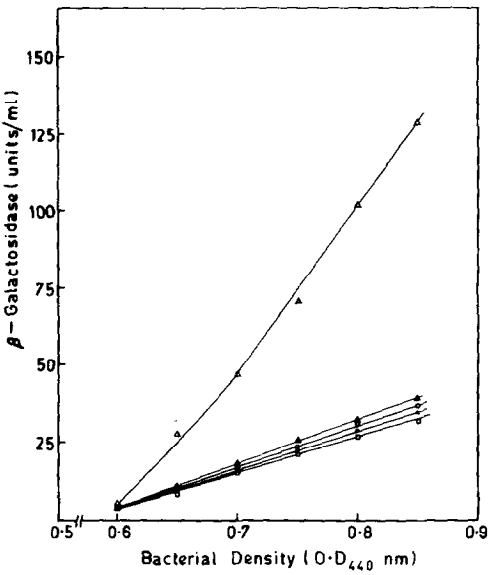


Figure 3. Effect of adenine, guanine and cyclic AMP on the inhibition of β-galactosidase. E. coli C was grown in TG medium and divided into five parts. The first was treated as the uninfected control. Each of the other parts was infected with ØX174 am E 3 at a moi = 6. Besides, the third part was treated with adenine while the fourth with guanine, to the fifth part was added cyclic AMP. All five parts were induced with IPTG. Aliquots were withdrawn at suitable intervals and assayed for β-galactosidase. △—△—△—△ uninfected control; ○—○—○—○ infected; ●—●—●—● infected plus adenine; ▲—▲—▲—▲ infected plus guanine; □—□—□ infected plus cyclic AMP.

(iii) Effect of Adenine, Guanine and Cyclic AMP on the synthesis of β-galactosidase in infected cells. In order to determine whether the inhibition was caused by depletion in nucleotide pools, adenine and guanine were respectively added to the growth media to a final concentration of 4 mM before the cells were infected with ØX174 am 3. It was seen that addition of these nucleotide bases had no effect whatever on the degree of inhibition (Fig. 3). The involvement of cyclic AMP, if any, in the repression process was also investigated in a similar manner. Cyclic AMP was added to the growth medium to a final

concentration of 4 mM and the induction was studied. No relaxation was observed in the degree of repression (Fig.3). These results strengthen the argument that neither nucleotide pools nor cyclic AMP are involved in the inhibition process.

DISCUSSION : Results of our experiments show that the synthesis of β-galactosidase is repressed in E. coli C when the cells are infected with ØX174 containing amber mutation in any of its nine genes except one. The argument that the repression is caused by a phage gene-specified product is thus further strengthened. Moreover, the exact phage gene, viz. gene A, associated with the repression phenomenon is identified. This product, which is an indispensable factor for RF DNA replication, has a role to play in the repression phenomenon as well.

The simultaneous absence of RF replication and the repression phenomenon in the case of amber A mutant might tempt one to suggest that the action of the cistron A product is indirect, i.e., it enables phage RF DNA to replicate, which in turn causes the repression. This could not be set aside offhand since the synthesis of phage DNA within the cell might lead to the depletion of nucleotide pools which in various ways could reduce the rate of enzyme synthesis. If such was the case, repression would not have occurred in cells which do not support RF replication of ØX174 mutants which may or may not produce the cistron A protein. E. coli C rep 3 is such a mutant which supports the conversion of viral single-stranded DNA to the double-stranded replicative form, but not the replication of RF (10). In such a mutant of E. coli, too, the synthesis of β-galactosidase was repressed to the same degree

as in cells which were able to replicate the RF DNA and the action of cistron A product was proved to be direct.

That the repression was not due to any depletion in nucleotide pools was also verified. Such depletion, if any, would have been made up for by the addition of nucleotide bases externally, so that nucleotides would be produced through salvage pathways and the rate of enzyme synthesis, if not restored to the normal level, would at least have shown considerable recovery. Hence, the observation that the inhibition of β-galactosidase synthesis was not in the least relaxed by the addition of either adenine or guanine leads one to discount the occurrence of any such depletion of nucleotide pools.

Similar observations were made regarding the role of cyclic AMP. It was earlier shown (2) that since the synthesis of catabolite-insensitive enzyme alkaline phosphatase is also repressed in ØX174 infected cells, cyclic AMP has perhaps no role to play in the ØX-induced inhibition of the catabolite-sensitive enzyme β-galactosidase. In the case of ultraviolet irradiated cells such inhibitory effect is reversed by the addition of cyclic AMP (11). It was thereby conjectured that UV-irradiation, like glucose, causes the loss of this nucleotide. In the case of ØX-induced inhibition, however, absence of stimulating action of exogenous cyclic AMP argues against the occurrence of catabolite repression.

Since ØX174 am A 62 does not cause repression while am A 8 does so, some interesting speculations may be made regarding the mechanism of ØX-induced repression. The cistron A, as it is now known, codes for two polypeptides of molecular weight 60,000 and 35,000 (4). Of the two, am A 62, which fails

to repress β-galactosidase synthesis, maps in the region that codes for both the polypeptides (3). The other mutant am A 8, however, synthesise the small A protein. Martin and Godson (12) had observed that mutants near the amino-terminal end of cistron A, which still produce the small 35,000 molecular weight polypeptide, shut-off host DNA synthesis while mutants near the carboxy-terminal end, which do not produce the small A polypeptide do not shut-off the host.

It is interesting to note that the ØX174 gene product which shuts-off host DNA synthesis predictably leads to the inhibition of host enzyme synthesis as well. Reasonably, it may be suggested that the small A protein of ØX174 perhaps acts on the same target, namely, the DNA molecule of the host, to bring about the cessation of DNA synthesis and the inhibition of enzyme synthesis.

In order to explain host DNA shut-off, Martin and Godson (12) have predicted that the small A protein possibly interacts with the host DNA at the single-stranded regions of replication forks, perhaps degrading the single-strands. The cistron A protein is reported to have endonucleolytic activity (13) and it is not yet clear whether this activity resides in the portion common to both large and small proteins. The small A protein has also been found to cleave the single-stranded viral DNA at different sites having, however, a preference for the origin of replication (14). Besides, only superhelical RF DNA, and neither relaxed covalently closed RF DNA nor RF II DNA, is a substrate for the endonucleolytic action (15).

Hence, it is not impossible that the small A protein attacks the supercoiled regions of the host DNA where a few

bases exist in unpaired state (16) and bring about conformational changes in these regions due to endonucleolytic cleavages. In that event, the rate of transcription of the DNA may be appreciably reduced. For there is evidence that the superhelical circular form of DNA has greater template efficiency for transcription than the nicked, relaxed form both *in vitro* (17) and *in vivo* (18). This leads us to suggest that reduction in the rate of transcription is at least one of the reasons for inhibition of enzyme synthesis of the host cell.

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