Regulation of the L-Arabinose Operon BAD in Vitro*

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SUMMARY

A DNA-directed cell-free system to study the L-arabinose BAD operon has been further developed so that the rate of synthesis of enzymes coded for by the structural genes in the operon in vitro is about 5% the in vivo rate. L-Arabinose isomerase and L-ribulokinase, the products of the araA and araB genes, are synthesized at the same rate, demonstrating that the operon is coordinately expressed in vitro. The system is completely dependent upon araC protein which can be supplied either by synthesis in vitro from an araC+ DNA template or by the addition of araC protein extracted from whole cells. The properties in the in vitro system of araC protein from either source suggests that the two products are similar if not identical. Both the repressor and activator forms of the araC protein are demonstrable in vitro using the appropriate templates confirming the essential aspects of the model for gene regulation of this operon.

The expression of the L-arabinose operon, araBAD, in Escherichia coli B/r is regulated by the protein product of the gene araC (2, 3). It has been shown that the araC protein can exist in two distinct functional forms, activator and repressor. The former, in the presence of L-arabinose, is required for expression of the operon while the latter prevents it (4). The model for regulation of the L-arabinose operon (3, 6) proposes that the transition from one functional state to the other is mediated by the inducer, L-arabinose. In the absence of L-arabinose the equilibrium between repressor and activator is in the direction of repressor with repressor bound to the ara operon (araO). In the presence of L-arabinose the equilibrium is shifted toward activator and the repressor is removed from araO. The activator functions at the initiator site (araI) to allow for expression of the operon. Measurement of ara mRNA produced in vivo suggests that both repressor and activator function at the level of transcription (7, 8, 9).

Superimposed upon the specific control exerted on the L-arabinose operon by the araC protein is a more general system of catab-

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‡ For the sake of simplicity we shall refer to this operon by its structural genes BAD to distinguish it from other operons, e.g. araE' concerned with the transport of L-arabinose and controlled by the araC gene(1). The araBAD operon is shown in Fig. 1.

olite positive control mediated through cyclic AMP⁸ and the catabolite gene activator (CGA) protein (10). The operon requires the presence of CGA protein and cyclic AMP for expression. Some recent experiments suggest that CGA protein interacts at a site which may be congruent with the araI site (11) but the relationship between CGA protein and the activator form of the araC protein is at present unclear.

Regulation of the L-arabinose operon in vitro has been studied using DNA-directed cell-free protein-synthesizing preparations. It has been demonstrated that some of the in vivo properties of the L-arabinose system are also present in an in vitro system which uses ara+ DNA and crude araC protein-containing extracts to stimulate synthesis of L-ribulokinase, the product of the araB gene (12). However, in a similar system using ara- DNA, the synthesis of L-ribulokinase has been observed without the addition of araC protein (13). The apparent conflict may have been clarified by the recent demonstration that araC protein can be supplied by de novo synthesis dependent upon the ara+ DNA template (14).

We have further developed the DNA-directed cell-free system to study the L-arabinose operon. We have defined optimum conditions for this system so that the rate of synthesis of L-arabinose isomerase per araA gene in vitro is about 5% of the in vivo rate and we can detect both L-arabinose isomerase and L-ribulokinase activities, the products of the araA and araB genes, respectively. The rate of synthesis of these two enzymes, expressed in terms of nanomoles of enzyme per μg of DNA per hour is very similar, demonstrating that the operon is coordinately expressed in vitro. The system is completely dependent upon araC protein which can be supplied either by synthesis in vitro from an araC+ DNA template or by the addition of araC protein extracted from whole cells. The increased sensitivity of the in vitro system has allowed us to compare the araC protein synthesized in the in vitro system with the properties of araC protein purified from whole cells. The similar behavior of the in vitro system of araC protein from either source strongly suggests that the two products are similar if not identical.

MATERIALS AND METHODS

Bacterial Strains

All of the strains used in this study are E. coli K12 strains which contain the ara region from E. coli B/r. It is necessary to transfer the ara region from B/r to K12 because the λ8043 phage used as a source of DNA is grown in K12 and such phage DNA would be destroyed by restriction enzymes in cell-free systems.

The abbreviations used are: cyclic AMP, cyclic adenosine 3'5'-monophosphate; CGA protein, catabolite gene activator protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
extracts prepared from B/r strains. SB7219(Δ755), SB7228(Δ719), and SB7223(Δ744) were constructed by PI transduction and are otherwise isogenic derivatives of strain 514 described by Zubay et al. (15). The ara deletions used in this study are shown in Fig. 1.

Phase Strain

\( \lambda \theta 80 \text{dara} \) phage is isolated, as previously described (7), from the doubly lysogenic strain SB7500(ara2Δ74) which contains \( \lambda \theta 80 \) and \( \lambda \theta 80 \text{dara} \). The ara region on the \( \lambda \theta 80 \text{dara} \) phage is from E. coli B/r. Otherwise isogenic derivatives of the \( \lambda \theta 80 \text{dara} \) phage have been constructed which contain various deletions (Fig. 1) in the l-arabinose region. araA766 was transferred to the \( \lambda \theta 80 \text{dara} \) phage by an F' Δ ara strain by P. Cleary, University of Minnesota. \( \lambda \theta 80 \text{dara} \) phage is isolated from SB7600 which is SB7219 lysoginized with \( \lambda \theta 80 \) and \( \lambda \theta 80 \text{dara} \). \( \lambda \theta 80 \text{dara} \) phage was constructed from a cross of a F' Δ ara strain with a phage was constructed from a cross of a F' Δ ara strain with a...

Fig. 1. The L-arabinose gene enzyme complex in Escherichia coli B/r. The horizontal line represents the segment of the genome which contains the genes whose products are involved in the catabolism of L-arabinose. Genes araA, araB, and araD code for the enzymes which convert L-arabinose to D-xylulose 5-phosphate. araC is the regulatory gene; araO is the operator; and araI is the site where araC protein interacts. The numbers represent mutations.

**Conditions for in Vitro Protein Synthesis**

The procedure used for the preparation of S30 extracts is similar to that described by Zubay et al. (15) except for the following modifications: cultures of SB7223 are grown at 30° in 2-liter Klyuyver flasks and harvested at an A600 of 2 with a yield of about 3.5 g of packed cells per liter of medium. The cells are immediately washed twice in Buffer I (0.01 M Tris, 0.014 M magnesium acetate, 0.06 M KCl, and 0.006 M 2-mercaptoethanol) and resuspended in Buffer II (0.01 M Tris-acetate, 0.014 M magnesium acetate, 0.06 M KCl, and 0.001 M dithiothreitol) at a ratio of 1 g of cells to 2 ml of buffer by passage through an Amino French pressure cell at a pressure of 1300 to 2000 psi. The final mixture volume is 0.15 ml. The synthesis was terminated by addition of 4 pg per ml of highly purified lac repressor (the generous gift of A. Riggs), chloramphenicol (100 pg per ml) or rifampicin (2 pg per ml). The inhibition observed with lac repressor is reversed by the presence of 6 X 10^-4 M IPTG. If the \( \lambda \theta 80 \text{dara} \) and the \( \lambda \theta 80 \text{dara} \) DNA are both present in the same synthesis mixture, one can detect both \( \beta \)-galactosidase and L-arabinose isomerase activities. Thus, we are able to distinguish between specific effects on the expression of the L-arabinose operon and more general effects on protein synthesis.

**Assay of L-Arabinose Isomerase (EC 5.3.1.4) and L-Ribulokinase (EC 2.7.1.16)**

L-Arabinose isomerase is assayed by the method of Cribbs and Englesberg (16). Assay mixture (0.1 ml or 0.15 ml) containing 0.4 M glycyglycine, pH 7.8, 0.1 M MnCl2, 1.0 M l-arabinose, and 200 pg per ml of chloramphenicol is mixed with an equal volume of protein synthesis mixture and incubated at 37° for a suitable length of time (5 to 60 min). The assay is stopped by the addition of 0.9 ml of 0.1 M HCl. The amount of L-ribulose produced is determined by the cysteine-carbazole test (17).

L-Ribulokinase is assayed by a radiometric method in which the conversion of l-[\( 1^4 \)C]ribulose to l-[\( 1^4 \)C]ribulose 5-phosphate is measured (18). Five microliters of chloramphenicol (2.5 pg per ml) and 5 \( \mu l \) of DNAse (200 pg per ml) are added to the protein synthesis mixture. After 5-min incubation at 37°, 5 to 10 \( \mu l \) of the resulting solution are added to 0.1 ml of L-ribulokinase reaction mixture containing 5.6 X 10^-6 cpn of l-[\( 1^4 \)C]ribulose and incubated for 16 hours at 30°. The assay is linear for this period of time.
system, further purified by DNA cellulose chromatography, or stored indefinitely in nitrocellulose or polypropylene tubes in a liquid nitrogen freezer.

**Step 2—**DNA cellulose is prepared according to the method of Alberts et al. (19), except that the ultraviolet irradiation step of Litman (20) was included to help the DNA adsorb to the cellulose. The product of “Step 1” is applied to a column (3.7 x 10 cm) containing 50 g of salmon sperm DNA cellulose (8 mg of DNA per g of cellulose) and washed on with 100 ml of Buffer B. The column is further washed with 300 ml of Buffer B containing 0.3 M potassium acetate before it is eluted with Buffer B containing 0.5 M potassium acetate. The fractions containing araC protein activity, as determined in the in vitro system herein described, are pooled, frozen in liquid nitrogen, and stored in a liquid nitrogen freezer where araC protein activity is stable for about 2 weeks.

**RESULTS**

**Activator Function of araC Protein in Vitro**—The cell-free DNA-dependent protein-synthesizing system is composed of an “S30” (cell-free extract) that contains ribosomes and all of the protein factors required for protein biosynthesis, DNA from defective transducing phages carrying the bacterial genes to be studied, and those cofactors and substrates necessary for RNA and protein synthesis. Our S30 is prepared from a strain of *E. coli* that contains deletions of both the L-arabinose and lactose genes and, therefore, devoid of any of the products of these systems. araC protein is obtained from a strain which contains a deletion of the araAB region. The DNA used to program the system contains a deletion of the araC gene to ensure that any activation of the operon results from the addition of araC protein and not from the synthesis of araC protein in the in vitro system. Thus, any detectable L-arabinose isomerase or L-ribulokinase (the products of the araA and araB genes) after the incubation is the result of a net in vitro synthesis stimulated by the addition of araC protein.

Crude araC protein added to the in vitro system stimulates L-arabinose isomerase synthesis at least 90-fold after a 60-min incubation (Table I). The amount of L-arabinose isomerase produced is proportional to the amount of *MsbDaraC*-DNA added in the range of 4 to 60 μg per ml (data not shown). The data presented in Table I also show that zero time for synthesis, the omission of L-arabinose, cyclic AMP, araC protein, or DNA, or the addition of n-fucose or chloramphenicol result in no synthesis of L-arabinose isomerase. The above results, all consistent with in vivo studies, suggest that the L-arabinose operon is being activated by the same mechanisms in vitro as it is in vivo.

The in vitro system can be used as an assay for araC protein activity in cell-free extracts. The results in Fig. 2 show that the amount of L-arabinose isomerase produced is proportional to the amount of added araC protein. Although the addition of 1 μg of araC protein purified by affinity chromatography as previously described (21) stimulates the synthesis of 1.8 x 10^3 monomers of L-arabinose isomerase per ml of synthesis mixture, the instability of araC protein purified by this procedure forced us to find an alternative method for purification (see “Materials and Methods”). Partially purified araC protein (1 μg) contained in a 20 to 50% ammonium sulfate cut results in the synthesis of 8 x 10^3 monomers of L-arabinose isomerase per ml of synthesis mixture and is very stable when stored in a liquid nitrogen freezer. The crude araC protein is further purified about 500-fold by chromatography on salmon sperm DNA cellulose. This product (1 μg) stimulates the synthesis of 2.4 x 10^3 monomers of L-arabinose isomerase per ml of synthesis mixture.

**Repressor Activity of araC Protein in Vitro**—Initiator constitutive (araT+) mutants have been described in strains containing deletions of the araC gene (6). These mutants have a cis-acting, partially constitutive phenotype. The enzyme levels of the mutants are repressed by the product of the araC gene in the absence of L-arabinose (repressor form) and further induced by the presence of araC gene product in the presence of L-arabinose (activator form). A mutant containing a double mutation in the initiator region (araT+I/) has recently been isolated (22). This mutant has a higher constitutive rate of ara operon expression corresponding to about 20% of the fully induced wild type level but responds to the activator and repressor forms of araC protein the same as the parent araT+.

The relative enzyme levels of the araT+I/ strains are shown in Table II.

If the in vitro system is programmed with a *MsbDaraC*-DNA template, L-arabinose isomerase is produced in the absence of araC protein and L-arabinose (Table II). The addition of both L-arabinose and araC protein results in a large increase in the amount of L-arabinose isomerase synthesized. However, the addition of araC protein to a reaction mixture containing no L-arabinose results in a significant reduction in the synthesis of L-arabinose isomerase (Table II). The repression is specific for the ara operon since the synthesis of β-galactosidase programmed by lac DNA is not affected by the addition of crude araC protein (data not shown). It can also be seen in Table II that the amounts of L-arabinose isomerase produced from the various DNA templates in vitro is proportional to the amount found in the corresponding in vivo situation.

**In Vitro Synthesis of araC Protein**—If the system is pro-

### Table I

**Enzymatic activities resulting from cell-free synthesis**

<table>
<thead>
<tr>
<th>Incubation system</th>
<th>L-arabinose isomerase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/μl</td>
</tr>
<tr>
<td>Complete</td>
<td>9.2</td>
</tr>
<tr>
<td>Complete at zero time for synthesis</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Complete, no DNA</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Complete, no L-arabinose</td>
<td>0.1</td>
</tr>
<tr>
<td>Complete, no araC protein</td>
<td>0.1</td>
</tr>
<tr>
<td>Complete, + 0.27 μM n-fucose</td>
<td>0.1</td>
</tr>
<tr>
<td>Complete, + 85 μg per ml of chloramphenicol</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

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grammed with \( \lambda h80dara^{+} \) DNA and no araC protein is added to the synthesis mixture, L-arabinose isomerase and L-ribulokinase activities are present after the incubation (Table III). The amount of L-arabinose isomerase produced is proportional to the amount of ara+ DNA added in the range of 4 to 40 \( \mu \)g per ml (data not shown). The data presented in Table III also show that zero time for synthesis, no DNA, or the addition of chloramphenicol result in no synthesis of L-arabinose isomerase. The synthesis of L-arabinose isomerase from the ara+ template is dependent upon the presence of L-arabinose and is inhibited by \( \alpha \)-fucose. Furthermore, cyclic AMP stimulates synthesis from the ara+ template. The above results, all consistent with in vivo studies, suggest that the L-arabinose operon is being regulated by the same mechanisms in vivo as it is in vitro. Therefore, araC protein must be synthesized in vivo and in the presence of L-arabinose activates the operon resulting in the synthesis of L-arabinose isomerase and L-ribulokinase.

Further support for the in vitro synthesis of araC protein is obtained from the complementation experiments presented in Table IV. Neither \( \lambda h80dara^{-} (\Delta 766) \) nor \( \lambda h80dara A^{-} B^{-} (\Delta 756) \) is capable of stimulating significant L-arabinose isomerase synthesis, but a mixture of the two DNAs behaves like ara+ DNA. This indicates that the araC+ B+ A+ template is

### Table III

**Synthesis of araC protein in vitro**

Table III was as described in the legend to Fig. 1 except that \( \lambda h80dara B^{+} A^{+} B^{+} (\Delta 756) \) DNA replaces the \( \lambda h80dara A^{+} B^{+} (\Delta 756) \) DNA and no araC protein is added. Enzymatic activities are assayed as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Incubation system</th>
<th>( \text{L-Arabinose isomerase} ) units/ml</th>
<th>( \text{L-Ribulokinase} ) units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>4.1</td>
<td>0.28</td>
</tr>
<tr>
<td>Complete at zero time for synthesis</td>
<td>&lt;0.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Complete, no DNA</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Complete, (-\lambda h80dara^{+}) DNA + (\lambda h80dara C^{+} (\Delta 766)) DNA</td>
<td>0.15</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Complete, (-\text{ara}^{+}) DNA + (\lambda h80dara C^{+} (\Delta 766)) DNA</td>
<td>0.35</td>
<td>0.4</td>
</tr>
<tr>
<td>Complete, + 0.27( \mu )m ( \alpha )-fucose</td>
<td>0.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Complete, + CAMP</td>
<td>0.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Complete, + 85( \mu )g/ml chloramphenicol</td>
<td>&lt;0.1</td>
<td></td>
</tr>
</tbody>
</table>

### Table IV

**Template complementation studies**

Protein synthesis was as described in the legend to Fig. 1 except that the DNA template is varied as indicated and no araC protein is added. The enzyme assays were incubated for 60 min as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>DNA</th>
<th>( \mu )g/ml</th>
<th>( \text{L-Arabinose isomerase} ) units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara+</td>
<td>38</td>
<td>4.1</td>
</tr>
<tr>
<td>ara+</td>
<td>38</td>
<td>0.15</td>
</tr>
<tr>
<td>ara+</td>
<td>38</td>
<td>0.7</td>
</tr>
</tbody>
</table>

### Table II

**Activation and repression in vivo and in vitro**

Protein synthesis was under standard conditions except that the DNA templates, L-arabinose, and araC protein are varied as indicated. All of the DNAs were present at a final concentration of 37 \( \mu \)g per ml. Five microliters of araC protein-containing solution from "Step 1" of the purification (see "Materials and Methods") containing 40\( \mu \)g per ml of protein is added just prior to the addition of the SS0 where indicated. When araC protein is present in a synthesis mixture containing no L-arabinose, the product of "Step 1" has been dialyzed against Buffer B without L-arabinose for 18 hours.

<table>
<thead>
<tr>
<th>Strain</th>
<th>L-Arabinose isomerase activity</th>
<th>System</th>
<th>L-Arabinose isomerase synthesized in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{in vivo} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ara+</td>
<td>+ara</td>
<td>( \lambda h80dara C^{+} (\Delta 766) ) DNA</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>ara+</td>
<td>+ara</td>
<td>( \lambda h80dara C^{+} (\Delta 766) ) DNA + araC protein</td>
<td>0.3</td>
</tr>
<tr>
<td>( \text{araC}^{+} (\Delta 766) )</td>
<td>-ara</td>
<td>( \lambda h80dara C^{+} (\Delta 766) ) DNA + araC protein</td>
<td>0.7</td>
</tr>
<tr>
<td>ara+</td>
<td>+ara</td>
<td>( \lambda h80dara C^{+} (\Delta 766) ) DNA + araC protein</td>
<td>2.2</td>
</tr>
<tr>
<td>ara+</td>
<td>+ara</td>
<td>( \lambda h80dara C^{+} (\Delta 766) ) DNA + araC protein</td>
<td>7.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>L-Arabinose isomerase activity</th>
<th>System</th>
<th>L-Arabinose isomerase synthesized in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara+</td>
<td>+ara</td>
<td>( \lambda h80dara C^{+} (\Delta 766) ) DNA</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>ara+</td>
<td>+ara</td>
<td>( \lambda h80dara C^{+} (\Delta 766) ) DNA + araC protein</td>
<td>0.3</td>
</tr>
<tr>
<td>( \text{araC}^{+} (\Delta 766) )</td>
<td>-ara</td>
<td>( \lambda h80dara C^{+} (\Delta 766) ) DNA + araC protein</td>
<td>0.7</td>
</tr>
<tr>
<td>ara+</td>
<td>+ara</td>
<td>( \lambda h80dara C^{+} (\Delta 766) ) DNA + araC protein</td>
<td>2.2</td>
</tr>
<tr>
<td>ara+</td>
<td>+ara</td>
<td>( \lambda h80dara C^{+} (\Delta 766) ) DNA + araC protein</td>
<td>7.1</td>
</tr>
</tbody>
</table>
The concentration of L-arabinose was 6.7 X 10^{-2} M. After incubation at 37°C for 60 min, the synthesis was terminated and the enzyme assay initiated by the addition of isomerase reaction mixture. O, araA^+ B^+ C^+ DNA template was present at a concentration of 37 µg per ml. Maximum activity corresponds to 1.3 X 10^5 monomers of isomerase per hour per ml of reaction mix per µg of DNA. X, araA^+ B^+ C^- DNA at a concentration of 38 µg per ml and 5 µl of crude araC protein were present in each synthesis mixture. Maximum activity corresponds to 2.3 X 10^5 monomers of isomerase per hour per ml of reaction mix per µg of DNA.

Fig. 4. Synthesis of L-arabinose isomerase in response to varying d-fucose concentrations. Synthesis was under standard conditions and the d-fucose concentration was varied as indicated. The concentration of L-arabinose was 6.7 X 10^{-2} M. After incubation at 37°C for 60 min, the synthesis was terminated and the enzyme assay initiated by the addition of isomerase reaction mixture. O, araA^+ B^+ C^+ DNA template was present at a concentration of 31 µg per ml. Maximum activity corresponds to 6 X 10^5 monomers of isomerase per hour per ml of reaction mix per µg of DNA. X, araA^+ B^- C^- DNA template at a concentration of 38 µg per ml and 5 µl of crude araC protein were present in each synthesis mixture. Maximum activity corresponds to 7 X 10^5 monomers of isomerase per hour per ml of reaction mix per µg of DNA.

The kinetics of synthesis of both L-arabinose isomerase and L-ribulokinase from an ara^+ DNA template have also been determined. At various times after the synthesis is initiated, chloramphenicol and DNase are added to stop the reaction and the synthesis mixture is diluted into the appropriate assay mixture. The monomers of enzyme synthesized are calculated from the specific activities of purified L-arabinose isomerase (12 units per µg of protein (23)) and L-ribulokinase (2.6 units per µg of protein). L-Ribulokinase appears before L-arabinose isomerase and the rate of synthesis of the two enzymes is almost identical, 1.8 X 10^5 monomers per hour per ml of synthesis mixture per µg of DNA.

DISCUSSION

The in vitro protein-synthesizing system can produce as many as 50 monomers of L-arabinose isomerase per araA gene per hour of incubation when araC protein purified on salmon sperm DNA cellulose is added. This estimate is based on a subunit molecular weight for L-arabinose isomerase of 60,000, a specific activity for the purified enzyme of 12 units per µg of protein, and a molecular weight of 30 X 10^6 for the phage DNA which contains one copy of the araA gene. At least 1 unit of L-arabinose isomerase per µg of DNA can be produced during a 1-hour incubation (Fig. 2).

N. Lee, personal communication.

able to provide a product, the araC protein, that is required for the synthesis of L-arabinose isomerase from the araC^-B^+ A^+ template. Thus, just as in vivo, L-arabinose isomerase synthesis is positively controlled by the product of the araC gene.

Comparison of in Vitro and in Vitro araC Protein—By comparing the system programmed with ara^+ DNA (in vitro araC protein synthesis) to a system containing araC^- DNA and added araC protein obtained from whole cells (in vivo araC protein) the two products can be compared.

The effects of L-arabinose and d-fucose on "in vivo" and "in vitro" araC protein have been determined. The induction experiment shown in Fig. 3 shows that the dependence of both systems on L-arabinose is quite similar. An apparent K_m of 2 X 10^{-3} M for the interaction of L-arabinose with both "in vivo" and "in vitro" araC protein may be estimated if we assume that induction of the operon reflects binding of L-arabinose to the araC protein. The data presented in Fig. 4 show the effect of the anti-inducer, d-fucose, on the expression of the L-arabinose operon. Once again a single curve can be drawn for both in vivo and in vitro araC protein, corresponding to an apparent K_i of 2 X 10^{-4} if we assume inhibition is the result of the binding of d-fucose to the araC protein. The experiments reported above strongly indicate that the araC protein synthesized in vitro is very similar to the in vivo product.

Kinetics of Synthesis—The time required to synthesize an inducing amount of araC protein in the in vitro system can be determined by measuring the time required for the first appearance of L-arabinose isomerase activity in the system programmed with ara^+ DNA compared to the time required in the system programmed with araC^- (Δ68) DNA in the presence of added araC protein. All of the components of the in vitro system except the S30 are mixed together and divided equally among 10 tubes. Synthesis is initiated by the addition of S30 and stopped at the indicated times by the addition of the L-arabinose isomerase assay mixture which contains chloramphenicol. In the system programmed with ara^+ DNA the first appearance of L-arabinose isomerase is at approximately 27 min, if one extrapolates to the time axis, and the synthesis continues at a constant rate until at least 60 min. If the system is programmed with araC^- (Δ68) DNA in the presence of added araC protein, L-arabinose isomerase activity first appears 14 min after the initiation of synthesis. The lag of 13 min reflects the time required to synthesize and assemble sufficient araC protein to activate the ara operon (Fig. 5).

Fig. 3. Synthesis of L-arabinose isomerase as a function of the L-arabinose concentration. Synthesis was under standard conditions except that the L-arabinose concentration was varied as indicated. After incubation at 37°C for 60 min, the synthesis was terminated and the enzyme assay initiated by the addition of isomerase reaction mixture. O, araA^+ B^+ C^+ DNA template was present at a concentration of 37 µg per ml. Maximum activity determined by measuring the time required for the first appearance of reaction mix per pg of DNA. X, araA^+ B^+ C^- DNA at a concentration of 38 µg per ml and 5 µl of crude araC protein were present in each synthesis mixture. Maximum activity corresponds to 6 X 10^4 monomers of isomerase per hour per ml of reaction mix per µg of DNA.
which corresponds to $1 \times 10^{10}$ monomers of L-arabinose isomerase from $2 \times 10^{10}$ molecules of $\lambda h80daraC^{-} DNA$.

The differential rate of synthesis of L-arabinose isomerase in a wild type strain (UP1000) growing with a generation time of 1 hour is 40 units per mg of protein. This corresponds to 3.3 $\mu$g or $3.3 \times 10^{10}$ monomers of L-arabinose isomerase per mg of protein. Since there is about 1 mg of protein in $10^{10}$ cells, there are $3.3 \times 10^{10}$ monomers of L-arabinose isomerase per cell. A cell in the exponential phase of growth contains 3 to 4 chromosomes each of which contains one araA gene. Thus, 1000 monomers of L-arabinose isomerase are produced per araA gene in vitro. Therefore, the number of monomers of L-arabinose isomerase produced per araA gene in vitro is approximately 5% (50/1000) of the number produced in vivo if we assume that specific activities of the in vivo and in vitro enzymes are the same. The rate of synthesis in vivo may be an underestimate since we are assuming that all of the DNA molecules added are active as templates.

The DNA-directed protein-synthesizing system used in this paper appears to be more efficient than those used in the past to study the regulation of the L-arabinose operon. The most important differences that resulted in a more efficient system are in the preparation of the S30. Processing the cells immediately rather than freezing them and the use of very low pressures when lysing the cells in the French press are of primary importance. The S30 is stable for at least 6 months when stored in liquid nitrogen.

The inclusion of polyethylene glycol in the synthesis mixture produces a 2- to 3-fold increase in the rate of enzyme synthesis. A similar effect has also been described in in vitro studies on the tryptophan operon (24). The assay for L-arabinose isomerase is much more convenient and rapid than the assay for L-ribulokinase. Since the two enzymes are coordinately produced, the induction of the operon is most easily monitored by assaying for L-arabinose isomerase activity.

The data shown in Fig. 2 demonstrate that the system is a quantitative assay for araC protein when it is programmed with araC- DNA. To routinely use the in vitro system as an assay it is necessary to standardize each DNA preparation and each S30 as small variations may occur. It is also necessary to determine the effect of a given sample on general protein synthesis by measuring $\beta$-galactosidase synthesis from a lac DNA template in the same synthesis mixture. Although the assay is tedious it has two advantages over the DNA binding assay previously described for the araC protein (21): one can detect araC protein activity in crude extracts and the biological activity of the protein is being measured.

The experiments reported in this paper demonstrate that many of the in vivo properties of the L-arabinose operon are also present in our in vitro system. We have shown that the in vitro system is absolutely dependent upon araC protein which can be supplied either by de novo synthesis from an araC+ DNA template or by the addition of araC protein purified from whole cells. araC protein can activate the operon from the trans position in vitro as demonstrated by the complementation experiments presented in Table III. In the absence of L-arabinose the araC protein has been shown to act as a repressor. The operon is expressed coordinately in the in vitro system and the appearance of the promoter proximal gene product, L-ribulokinase, precedes the first appearance of the more distal gene product, L-arabinose isomerase.

We find, as have others (12, 13, 14), that activation in vitro requires L-arabinose and cyclic AMP and that $\alpha$-fucose inhibits activation in the presence of L-arabinose. We have compared the araC protein made in vitro with araC protein purified from whole cells and find the two products to be very similar—the apparent $K_m$ for L-arabinose and $\alpha$-fucose is $2 \times 10^{-4}$ M for both products. It has been shown that in vitro the L-arabinose operon is half-induced at an intracellular concentration of $6 \times 10^{-10}$ M L-arabinose (25). The interaction of L-arabinose and $\alpha$-fucose with electrophoretically pure araC protein has been studied by fluorescence techniques. An apparent $K_m$ of $3 \times 10^{-10}$ M for L-arabinose and $6 \times 10^{-10}$ M for $\alpha$-fucose was found. Thus, a relative high concentration of the inducer, L-arabinose, or the anti-inducer, $\alpha$-fucose, is required before binding to the araC protein occurs and the results obtained both in vivo and in vitro are very similar.

The isolation of the araA* mutants has allowed us to develop an assay for the repasser form of araC protein using the in vitro system. The constitutive synthesis of L-arabinose isomerase which is observed in vivo in araA* C- strains is also observed in vitro when the system is programmed with araA* C+ DNA. The rate of synthesis of L-arabinose isomerase both in vivo and in vitro is decreased if araC protein is present in the absence of L-arabinose.

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