Arabinose C Protein: Regulation of the Arabinose Operon *in vitro*

JACK GREENBLATT

Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

ROBERT SCHLEIF

Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154

The detection of the gene *ara* C protein using a DNA-dependent *in vitro* protein synthesizing system represents the first isolation of an operon specific positive regulator with an *in vivo* role that has been genetically defined.

THE protein product of the gene *ara* C is required for normal expression of the L-arabinose operon in *Escherichia coli*[1]. If the C protein is absent, either because of deletion of the C gene or a nonsense mutation within it[2], the arabinose operon cannot be induced. The C protein is therefore a positive regulator of the operon. C gene mutants known as C^c have also been isolated which have high constitutive levels of arabinose enzymes in the absence of L-arabinose[3]. Cells containing a C^+ gene in addition to a C^c gene are not constitutive[1]. From this and other observations it was concluded that, in addition to its positive control properties, C protein also has repressing properties[4]. The biochemical basis for these control functions remains unknown.

Here we report the detection of the gene *ara* C protein using a DNA-dependent *in vitro* protein-synthesizing system in which ribulokinase, an enzyme of the arabinose operon, is synthesized subject to both the positive and the negative controlling influences of exogenously added C protein. Furthermore, the ribulokinase synthesis is L-arabinose and 3′,5′-cyclic AMP dependent and is inhibited by D-fucose, as predicted from the *in vivo* behaviour of the operon. Further purification and a more detailed study of the arabinose C protein are now possible.

The C Protein Assay

The arabinose C protein is a regulatory protein possessing no known enzymatic activity to guide its purification. The *in vivo* behaviour of the arabinose operon makes it likely that C protein binds L-arabinose and also suggests that it may bind to DNA or to RNA polymerase. Nonetheless, our attempts to detect C protein using these properties have not been successful. We therefore turned to the ability of C protein to induce the synthesis of arabinose operon enzymes. The cell-free DNA-dependent *in vitro* protein-synthesizing system developed by Zubay and his collaborators synthesizes enzymatically active β-galactosidase[5]. This system requires the addition of lac operon DNA and responds appropriately to lac repressor, galactoside inducers and 3′,5′-cyclic AMP. We have used the system they have described to identify the action of C protein.

DNA containing the arabinose operon and a sample to be tested for C protein activity were added to a cell-free system containing no arabinose C protein and no arabinose operon enzymes. After 1 h of protein synthesis, we assayed for the production of ribulokinase, an enzyme coded by the operator proximal gene of the arabinose operon (*ara* B).

To assay the small quantities of active enzyme synthesized in an *in vitro* system it is necessary to have a sensitive assay and to begin with a system devoid of the enzyme. Ribulokinase transfers a phosphate from ATP to ribulose. If radioactive ribulose is used as a substrate, the amount phosphorylated can be measured by precipitating and counting the ribulose phosphate product[6]. In our assay conditions the rate of synthesis of product is constant for 4 h, after which it slowly decreases. When the incubation time is less than 15 h, the assay response is linear with concentrations of enzyme up to 0.04 μg. The assay has a sensitivity of 0.002 μg corresponding to about 5×10^9 dimeric ribulokinase molecules. The requirement for a system devoid of ribulokinase was met by using as a source of cell-free extract in the protein synthesis system cells deleted for the arabinose operon so that no arabinose enzymes whatsoever could be present.

The activity of the protein-synthesizing system was monitored by measuring its ability to synthesize β-galactosidase. This required that the protein-synthesizing extract be prepared from a strain with deletions in both the arabinose and lactose genes. The *lac* operon DNA used was that located on the transducing phage λ $plac_5$ (ref. 7).

The transducing phage λdara was used as a source of arabinose operon DNA because its genome is a hundred times more concentrated in the operon than is *E. coli* DNA. We had already shown that in λdara the arabinose operon and the gene *ara* C have replaced some of the phage late genes and that they are transcribed and translated under the control of the system which controls the late genes[8,9]. Thus extracts prepared from cells in which this phage is growing should be a rich source of C protein. These extracts must not, however, contain any ribulokinase. A point mutation was therefore put into the ribulokinase gene of the λdara phage and it was grown in cells deleted of their chromosomal arabinose genes. By these means we obtained the essential ingredients for an *in vitro*

assay of C protein: a rich source of C protein uncontaminated by ribulokinase, a source of arabinose operon DNA, and a system with which to attempt its transcription and translation into enzymatically active ribulokinase.

Induction of Ribulokinase Synthesis

Ribulokinase is synthesized when C protein-containing extract, L-arabinose, and 3′,5′-cyclic AMP are added to the cell-free protein synthesizing system described above and defined explicitly in the legend to Fig. 1. The background level of the ribulokinase assay was measured by including chloramphenicol or omitting λdara DNA during the *in vitro* synthesis. All data shown are the actual counts measured without subtraction of the very reproducible background. Detectable ribulokinase synthesis requires the presence of C protein (closed circles in Fig. 1), and is completely inhibited by the addition of chloramphenicol (open circles in Fig. 1). The synthesis is dependent upon the addition of λdara DNA, the response being linear with added DNA up to at least 50 μg/ml. (data not shown). Ribulokinase synthesis is routinely obtained and the amount of ribulokinase synthesized varies from 0.1 to 0.3 μg/ml. of *in vitro* synthesis reaction.

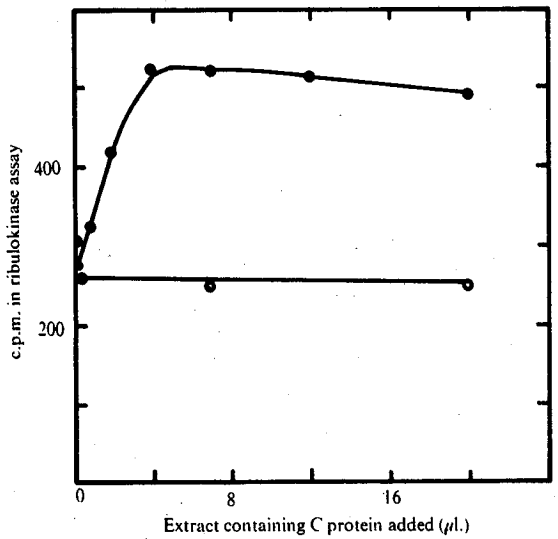

Fig. 1 *In vitro* synthesis of ribulokinase in the presence (○) and in the absence (●) of chloramphenicol as a function of the amount of added extract containing C protein. Cells deleted of their chromosomal arabinose region and lysogenic for a heat-inducible lysis defective λdara C⁺B⁻ prophage were grown in yeast-tryptone medium[9] at 34° C to a cell density of 5×10^8/ml. After a 10 min induction at 42° C and a further 80 min growth at 34° C, the cells were collected by centrifugation, ground with alumina, taken up in a minimal volume of buffer A (50 mM Tris-acetate, *p*H 7.4, 200 mM KCl, 10 mM Mg acetate, 0.1 mM K-EDTA, 0.1 mM dithiothreitol, 5% glycerol) and centrifuged 1 h at 100,000*g*, all manipulations being at 4° C. The supernatant was dialysed for 2 h against 100 volumes of buffer B (10 mM Tris-acetate, *p*H 7.8, 100 mM K acetate, 14 mM Mg acetate, 0.1 mM K-EDTA, 0.1 mM dithiothreitol) and then used to stimulate *in vitro* synthesis. The *in vitro* synthesis mixture was as described by Zubay[5]. The crude S-30 fraction was prepared from a strain JG148 containing both lactose and arabinose chromosomal deletions, and the preparation involved only one centrifugation step after grinding with alumina. *In vitro* synthesis of β-galactosidase using λplac$_5$ DNA as a template at 50 μg/ml. produced an A_{420} after 20 h incubation of 3 to 10 in the β-galactosidase assay described by Zubay[5]. Ribulokinase syntheses to assay C protein contained, in a total volume of 100 μl., 5 μg λdara DNA, 0.4 μM L-arabinose, 0.2 μM 3′,5′-cyclic AMP (Calbiochem), and, where indicated, 10 μg chloramphenicol. The protein concentration of the C protein extract was 25 mg/ml. as determined by the Biuret method using bovine serum albumin as a standard. After 1 h of protein synthesis at 37° C, using gentle shaking, 50 μl. of each reaction was mixed with 50 μl. of ribulokinase assay mixture (10 mM Mg acetate, 1 mM K-EDTA, 20 mM NaF, 15 mM GSH, 20 mM ATP, 10 mM NaN_3, 200 mM K phosphate, *p*H 7.8, 500 μg/ml. chloramphenol, 1 mg/ml. streptomycin and 20 mM ^{14}C-L-ribulose at 10^4 c.p.m./μM) and incubated at 37° C for 12 h. Into each tube were mixed 50 μl. of 200 mM Na pyruvate, 200 mM glucose-6-phosphate, 100 μl. of 1 M Ba acetate, and 1 ml. of absolute ethanol. After 30 min at 4° C the precipitates were collected on glass fibre filters (Reeve Angel 934AH). The filters were washed six times with 1.5 ml. cold 80% ethanol and counted in Aquasol (New England Nuclear). The background has not been subtracted from the ribulokinase assay c.p.m. C protein containing extract stimulates ribulokinase synthesis only in the absence (●) and not in the presence (○) of chloramphenicol.

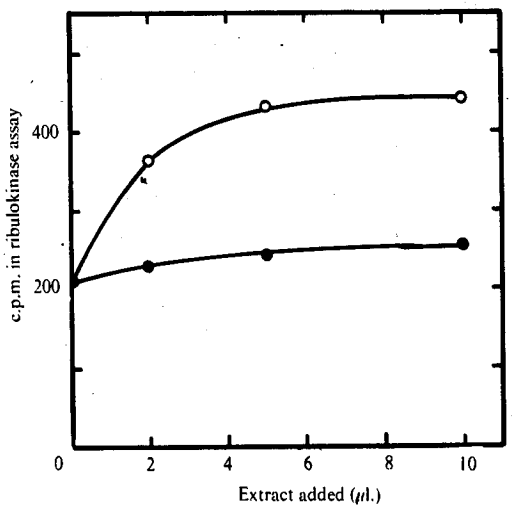

Fig. 2 The stimulation factor is not coded by a phage gene. Extracts were prepared and *in vitro* protein synthesis as described in the legend to Fig. 1 except that the reaction volumes were 50 μl. Ribulokinase assays were incubated for 14 h. Extracts used to stimulate *in vitro* ribulokinase synthesis were prepared from arabinose operon-deleted cells growing one of the heat-inducible lysis-defective phages λhy80 (●) or λdara C⁺B⁻ (○). Only the extract prepared from cells growing λdara C⁺B⁻ stimulates ribulokinase synthesis. The small amount of stimulation observed with λhy80 is also often seen with λdara when chloramphenicol is used to prevent synthesis, and is caused by some background ribulokinase activity present in ara B⁻ extracts.

The preparation of the C protein containing extract used to stimulate ribulokinase synthesis was different from that of the arabinose deletion crude extract used in the *in vitro* system (see legend to Fig. 1). The C protein extract was dialysed for a much shorter time at 4° C and it was not pre-incubated for 1 h at 37° C. It was therefore possible that the stimulation shown in Fig. 1 could be caused by some small molecule or by some macromolecule other than C protein. In addition, the stimulation could be effected by some regulator of phage lambda since the C protein-containing extract was prepared from cells growing λdara and the template arabinose operon DNA is located within λdara DNA. All these possibilities were largely excluded, however, by testing an extract prepared from cells growing λhy80, the phage most closely related to λdara[8,10]. That this extract does not stimulate ribulokinase synthesis, even though one prepared exactly in parallel from cells growing λdara does (Fig. 2), suggests that the product of a gene of the λdara phage is required for ribulokinase synthesis and that the implicated gene is not a phage gene.

Proof that C Protein is Involved

If the C protein controls ribulokinase synthesis, then a mutant C protein should have similar altered properties in *in vivo* and *in vitro* systems. A characteristic *in vivo* property of the L-arabinose system of *E. coli* is the interaction of D-fucose, an analogue of L-arabinose, with C protein that antagonizes induction of the arabinose operon[11]. C^c mutations can be found which lie within the C gene and which allow cells to grow on L-arabinose in the presence of D-fucose[3]. C^c mutants usually seem to be constitutive *in vivo*, but it was necessary to include L-arabinose in *in vivo* experiments with mutant C^c protein because, as we shall mention in more detail

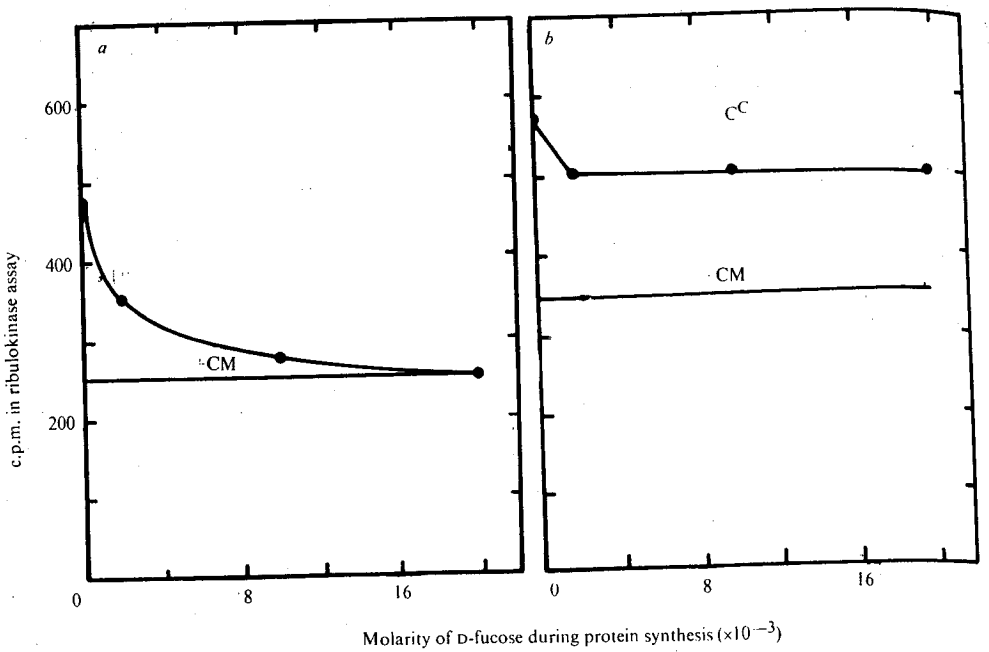

Fig. 3 Proof that it is a protein coded by *ara* C that is stimulating ribulokinase synthesis. 50 µl. *in vitro* synthesis reactions contained 200 µg of C protein containing extract prepared from cells growing either the phage λdara C^+B^- (*a*) or the phage λdara C^cB^- (*b*). Synthesis and assay conditions were as described in the legend to Fig. 1, except that synthesis reactions contained the indicated amounts of α-D-fucose (Sigma). Assay backgrounds were defined by running synthesis reactions in the presence of chloramphenicol. The background is somewhat higher in (*b*) than in (*a*) because the phage λdara C^cB^- makes a small amount of ribulokinase during its growth. Ribulokinase assays were incubated for 6.5 h. Only C^+ protein dependent ribulokinase synthesis is sensitive to D-fucose inhibition.

later, C^c protein does not induce either *in vitro* or *in vivo* in the absence of L-arabinose or some other inducer. An extract containing C^c protein was derived from cells producing C^c ribulokinase-negative λdara phage.

As expected from the *in vivo* behaviour of C^+ cells, addition of D-fucose to a system containing wild type C protein and L-arabinose completely depresses ribulokinase synthesis (Fig. 3*a*). In the crucial experiment, however, ribulokinase synthesis stimulated by mutant C^c protein in the presence of L-arabinose is resistant to D-fucose inhibition (Fig. 3*b*). The corresponding *in vivo* data on the behaviour of these C gene alleles are shown in Table 1. In agreement with the *in vitro* data, the C^c allele is D-fucose resistant while the C^+ allele is normally inducible and D-fucose-sensitive. This correspondence proves that the λdara gene whose product is essential for ribulokinase synthesis *in vitro* is the *E. coli* gene *ara* C.

Table 1 Properties of C^+ and C^c Alleles used for *in vitro* Studies

ara C allele	0.5% L-arabinose	1% D-fucose	Monomers isomerase per cell × 10^3
C^+	−	−	0.06
C^+	+	−	5.0
C^+	+	+	0.09
C^c	−	−	4.4
C^c	+	−	10.0
C^c	+	+	8.5

Arabinose isomerase levels in arabinose deletions containing λdara B^+ prophage. These strains are spontaneous revertants to ara^+ of the strains containing the prophage λdara C^+B^- and λdara C^cB^- which were used in the *in vitro* experiments described in the text. The cells were grown in minimal medium containing 1% glycerol and 1% casamino-acids. Arabinose isomerase was assayed as described previously[11]. The C^+ allele is L-arabinose inducible and D-fucose sensitive, while the C^c allele is constitutive and D-fucose resistant. Note that the C^c allele is induced to a higher enzyme level by L-arabinose.

In vitro Regulation

C protein-dependent ribulokinase synthesis *in vivo* requires the presence of an inducing amount of L-arabinose[12]. The corresponding *in vitro* induction experiment is shown in Fig. 4. Ribulokinase synthesis is completely L-arabinose dependent, requiring for half maximal induction an L-arabinose concentration of 10^{-3} M. Thus the *in vitro* system has the characteristic *in vivo* property of being induced by L-arabinose.

It has been shown that an intracellular L-arabinose concen-

arabinose operon of *E. coli* K12 (ref. 12). The apparent *Km*s observed *in vitro* and *in vivo* could depend on the relative concentrations of C protein and C protein sites of action (DNA or, possibly, RNA polymerase). Also, the gene *ara* C of the phage λdara is derived from *E. coli* $B/r^{8,10}$, and the C protein of this strain of *E. coli* might have an even higher *Km* for L-arabinose than *E. coli* K12. Therefore the apparent *Km*s measured *in vivo* and *in vitro* cannot yet be directly compared. The relatively high concentration of L-arabinose required for induction explains why it has not been possible to detect C protein by the radioactive sugar binding method used by Gilbert and Müller-Hill to detect lac repressor[13].

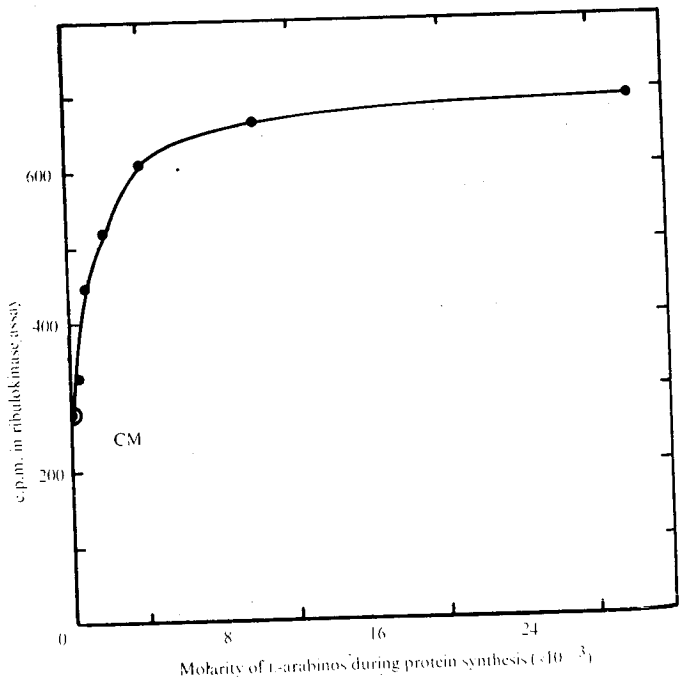

Fig. 4 Induction by L-arabinose of C protein-dependent *in vitro* ribulokinase synthesis. Synthesis and assay conditions were as described in the legend to Fig. 1, except that the L-arabinose concentration was varied as indicated. Each synthesis reaction contained 250 µg extract protein prepared from cells growing λdara C^+B^-. Each synthesis reaction was dialysed for 1.5 h against 200 volumes of buffer B so as to remove excess L-arabinose that would dilute the radioisotope used in the ribulokinase assay. The assays were incubated at 37° C for 13 h. The assay background is defined by the c.p.m. obtained when synthesis has been blocked by chloramphenicol.

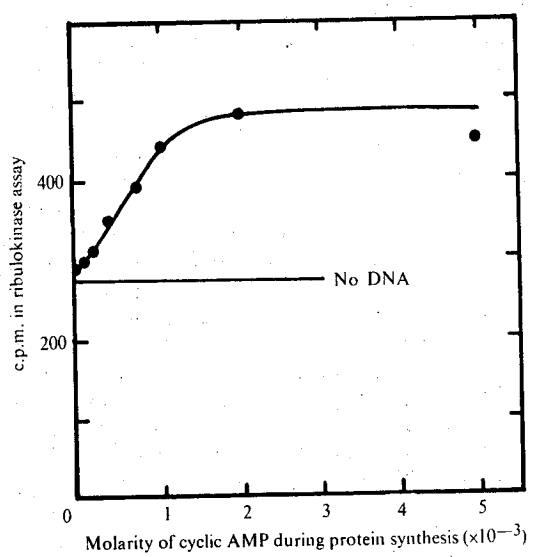

Fig. 5 Catabolite repression of C protein-dependent *in vitro* ribulokinase synthesis. Synthesis and assay conditions were as described in the legend to Fig. 1, except that the synthesis volumes were 50 µl. and the concentration of 3′,5′-cyclic AMP was varied as indicated. Each synthesis reaction contained 125 µg extract protein prepared from cells growing λdara C^+B^-, and the ribulokinase assays were incubated for 7 h. The assay background was defined as the average c.p.m. of two synthesis reactions lacking λdara DNA. Ribulokinase synthesis is stimulated at least ten-fold by the addition of a saturating amount of 3′,5′-cyclic AMP.

A second experiment prompted by the *in vivo* behaviour of the arabinose operon is to show that the C protein-dependent ribulokinase synthesis requires the presence of 3′,5′-cyclic AMP. The arabinose operon of λdara is catabolite repressible *in vivo*[9], implying that CAP factor[14] and 3′,5′-cyclic AMP[15] are required for full expression of the operon. The *in vitro* protein synthesizing system already contains CAP factor, but the omission of 3′,5′-cyclic AMP from the system should reduce or abolish ribulokinase synthesis. Fig. 5 shows the effect of restoring 3′,5′-cyclic AMP (chromatographically checked before use) to the system. Ribulokinase synthesis is stimulated at least ten-fold at saturation. The concentration of 3′,5′-cyclic AMP giving half maximal stimulation is approximately 5×10^{-4} M, ten times higher than that reported most recently for the lac system[5], but about the same as the value reported in a preliminary communication[16]. The inhibition of ribulokinase synthesis by D-fucose (Fig. 3*a*) and the dependence of synthesis on 3′,5′-cyclic AMP and L-arabinose provide strong evidence that the arabinose operon is being regulated correctly *in vitro*.

In vitro Repression by C Protein

Genetic data have suggested that, in addition to its inducing properties, C protein can also function as a repressor of arabinose enzyme synthesis[4]. If a cell is diploid for its arabinose region with genotype C^+/C^c, the phenotype is C^+ (inducible and D-fucose sensitive) rather than C^c (constitutive and D-fucose resistant)[1]. We have exploited this dominance of D-fucose sensitivity to test for C^+ repressor activity. C^c protein was used to stimulate ribulokinase synthesis in the presence of an inducing amount of L-arabinose. As was previously shown in Fig. 3, this synthesis is completely resistant to D-fucose. But as increasing quantities of C^+ protein were added and the *in vitro* system approached the equivalent of the C^+/C^c diploid state, the synthesis became increasingly sensitive to D-fucose inhibition (Fig. 6). Thus the C^+ phenotype is dominant to C^c, and the repressing properties possessed by C^+ protein *in vivo* are also elicited *in vitro*. The corresponding effects *in vivo* and *in vitro* of L-arabinose and D-fucose (Figs. 3, 4 and 6) confirm that *in vitro* induction and repression are a result of C protein action.

New Property of C Protein

The D-fucose resistant *ara* C^c mutants are usually constitutive with variable levels of arabinose operon enzymes in the absence of L-arabinose[3]. It was a surprise, therefore, to discover that C^c protein prepared from the one high level C^c strain we have tested depends completely on the presence of L-arabinose for its *in vitro* induction of ribulokinase synthesis. The concentration of L-arabinose giving half maximal induction with C^c protein is about 10^{-3} M, the same as with wild type C protein.

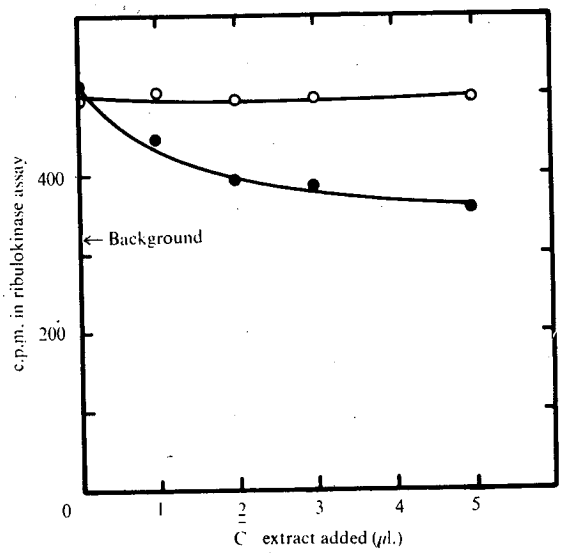

Fig. 6 *In vitro* repression by C^+ protein. Synthesis and assay conditions were as described in the legend to Fig. 1 except that synthesis volumes were 50 µl. Each synthesis reaction contained 110 µg extract protein derived from a strain growing the phage λdara C^cB^-. Increasing quantities of C^+ protein containing extract, up to 110 µg protein, were included in *in vitro* synthesis reactions run in the absence (○) and in the presence (●) of 10 mM D-fucose. Ribulokinase assays were incubated for 11 h. Assay background was defined by running synthesis reactions in the presence of chloramphenicol. In the presence of C^+ protein D-fucose sensitivity becomes dominant to D-fucose resistance.

This result suggested that, at least for some D-fucose resistant *ara* C^c mutants, the changed C^c protein can induce *in vivo* in the absence of L-arabinose, not because it is frozen in an inducing conformation, but because it is induced by some metabolite always present in growing cells. In an effort to find the unknown metabolite we tested various carbohydrates for ability to induce the C^c protein *in vitro*. Among those tested were L-arabinose, D-fucose, D-galactose, D-glucose, D-ribose and D-xylose. The only *in vitro* inducer of C^c protein we found, besides L-arabinose, was D-fucose, and D-fucose is probably not a naturally occurring compound in *E. coli*.

After this unexpected *in vitro* behaviour of C^c protein was discovered, we examined the *in vivo* induction properties of several independently isolated D-fucose resistant *ara* C^c mutants (Table 2). The four low level C^c mutants tested are induced by L-arabinose or D-fucose to higher arabinose enzyme levels. The two high level C^c mutants tested, including the λdara C^cB^- used for *in vitro* studies, seem to be induced to a higher level only by L-arabinose and not by D-fucose. The data suggest that D-fucose does not induce as efficiently as either L-arabinose or the postulated natural inducing metabolite. Nevertheless, it is clear that in these mutants the structure of

C protein is modified so that a ligand, D-fucose, which inhibits the wild type C^+ protein induces the mutant C^c proteins. Although it is only useful for *ara* C^c mutants, D-fucose is the only known gratuitous inducer of the arabinose operon.

We have used the cell-free DNA-dependent protein-synthesizing system developed by Zubay[5] to synthesize ribulokinase, an enzyme of the L-arabinose operon of *E. coli*. Ribulokinase synthesis *in vitro* requires the presence of DNA containing the arabinose operon, 3′,5′-cyclic AMP, L-arabinose, and the protein product of the gene *ara* C. The omission of any one of these results in little or no ribulokinase synthesis. We have therefore developed an assay for biologically active C protein with a system which can also be used to study the properties of C protein regulation of arabinose gene function.

Together with the dependence of ribulokinase synthesis on added λdara DNA the experiments shown in Figs. 1–6 demonstrate that the observed ribulokinase synthesis is *de novo* synthesis and not an artefact caused, for example, by microbial growth during the ribulokinase assays. Furthermore, because two independently isolated *ara* B^- mutants, one of them an amber mutant (Table 2), have been used in these experiments as a source of C protein (Fig. 3), the observed synthesis is unlikely to reflect an unusual property of the particular *ara* B^- mutant used.

Table 2 D-Fucose and L-Arabinose Induction of D-Fucose Resistant Constitutives

Strain	Monomers isomerase per cell × 10^4 Broth	Broth $+2\times10^{-2}$ M L-arabinose	Broth $+2\times10^{-3}$ M D-fucose
Wild type K-12 Hfr H	<0.1	2.3	<0.1
High level C^c	1.2	2.3	1.0
Low level C^c-1	<0.1	2.7	0.9
Low level C^c-2	0.15	2.7	1.2
Low level C^c-3	<0.1	2.5	0.8
Low level C^c-4	<0.1	2.5	1.0
Wild-type dara C^+B^-	0.01	2.1	0.01
High level dara C^cB^-	0.23*	0.49	0.10

Arabinose isomerase level (monomers per cell) in cells growing in broth plus the indicated sugars. All constitutives were derived from the wild type parent by nitrosoguanidine mutagenesis and grow at normal rates. The constitutives are fully D-fucose-resistant in arabinose minimal medium. D-Fucose does not induce as well as L-arabinose. The D-fucose was examined chromatographically and found to be free of L-arabinose (< 5%).

* The B^- mutation in this phage is an amber mutation, suppressible by su^+_{III} and 90% polar on the expression of the *ara* A (isomerase) gene.

The experiments depicted in Figs. 1–6 show that most of the known *in vivo* properties of C protein regulation also occur in the *in vitro* system. L-Arabinose is an inducer of the arabinose operon *in vivo*, and the same is found *in vitro*. D-Fucose, an antagonist of L-arabinose induction *in vivo*[11], also antagonizes induction *in vitro*. Furthermore, C^c protein prepared from a strain resistant to D-fucose inhibition *in vivo* is insensitive to D-fucose inhibition *in vitro*. This last fact proves that the system is indeed responding to C protein, for the C^c mutation is located within the gene *ara* C. Finally, in agreement with the fact that the arabinose operon is catabolite repressible *in vivo*[9], the *in vitro* synthesis of ribulokinase is dependent upon added 3′,5′-cyclic AMP.

Genetic evidence has suggested that C protein can function *in vivo* as either a repressor or an inducer[1,4] and this same functional duality has been demonstrated *in vitro*. In addition our *in vitro* experiments suggest that at least some D-fucose resistant *ara* C^c mutants have constitutive synthesis of arabinose enzymes as a result of an increased sensitivity of mutant C^c protein to some inducing metabolite always present in growing cells. In many of these D-fucose resistant mutants, C protein is modified so that D-fucose itself is an inducer, in sharp contrast to the properties of wild-type strains.

In recent years several negative regulators have been isolated, among them the lac repressor[13] and the repressors of phages λ (ref. 17) and 434 (ref. 18). These act to prevent messenger transcription when bound to homologous operator DNA[19,20]. In the presence of galactoside inducers the lac repressor detaches from the lac operator and allows transcription to proceed into the *lac* operon[21,22]. The mode of action of positive regulators, however, is much less clear. Several general positive-acting elements involved in the transcription process have been studied. Sigma factor is required for specific initiation of RNA synthesis by RNA polymerase[23] and is therefore a positively acting control element. The same is true of CAP factor, a protein which binds 3′,5′-cyclic AMP[14] and is required for messenger synthesis from the catabolite repressible operons[19]. The factor ψ_r is similar to CAP in that it also stimulates, at least *in vitro*, the synthesis of a specific class of RNA molecules, in this case ribosomal RNA[24]. The detection of C protein represents the first isolation of an operon specific positive regulator with an *in vivo* role which has been genetically defined. Although our initial attempts to purify C protein have not been successful, it should soon be possible to complete the purification and to study the biochemical basis of C protein control.

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