Environ. Pathol. Toxicol. 2, 1511-1527.
Thomas, J. C., Frey, C. M., & Stuehr, J. E. (1980a) *Inorg. Chem. 19*, 501-504.
Thomas, J. C., Frey, C. M., & Stuehr, J. E. (1980b) *Inorg. Chem. 19*, 505-510.
Tsangaris, J. M., Chang, J. W., & Martin, R. B. (1969) *Arch. Biochem. Biophys. 103*, 53-58.
Tsapakos, M. J., Hampton, T. H., & Jennette, K. W. (1981) *J. Biol. Chem. 256*, 3623-3626.
van Soestbergen, M., & Sunderman, F. W., Jr. (1972) *Clin. Chem. (Winston-Salem, N.C.) 18*, 1478-1484.
Wetlaufer, D. B. (1962) *Adv. Protein Chem. 17*, 303-390.
Yamashiro, S., Gilman, J. P. W., Hulland, T. J., & Abandowitz, H. A. (1980) *Acta Pathol. Jpn. 30*, 9-22.
Yarita, T., & Nettesheim, P. (1978) *Cancer Res. 38*, 3140-3145.
Zwelling, L. A., Anderson, T., & Kohn, K. W. (1979) *Cancer Res. 39*, 365-369.

Hyperproduction of *araC* Protein from *Escherichia coli*†

Robert Ferber Schleif* and Margaret Anne Favreau

ABSTRACT: Hypersynthesis of *araC* protein from *Escherichia coli* has been accomplished. The *araC* gene was cloned on plasmid pBR322, and some of the noncoding DNA preceding the *araC* gene was removed by exonuclease digestion. Finally, a DNA fragment containing the *lac* promoter and ribosome binding site was placed in front the *araC* gene. By these means the level of *araC* protein was increased about 5000-fold above the levels found in wild-type cells. This level of protein permits straightforward purification of sizeable quantities of *araC* protein.

The arabinose operon of *Escherichia coli* is positively and negatively regulated by the protein product of the *araC* gene (Greenblatt & Schleif, 1971; Wilcox et al., 1974; Sheppard & Englesberg, 1967; Englesberg et al., 1969). *araC* protein can bind to DNA and act as a repressor; however, when arabinose is present, *araC* protein can also act as in inducer by binding to a different DNA site (Ogden et al., 1980). As a consequence of the latter binding, RNA polymerase is able to bind to DNA, and transcription of the *araBAD* genes proceeds at a high rate.

In the past, the study of *araC* protein has been hampered by the low numbers of molecules which are present in cells, about 10 per cell, the difficulty of assaying, and the instability of *araC* protein (Greenblatt & Schleif, 1971; Steffen & Schleif, 1977a,b). The problems have been partly alleviated by the fusion of *araC* to the late gene promoter of phage λ or to a copy of the *lac* promoter carried on a plasmid. Both of these fusion products yield about a 50-fold overproduction of C protein (Steffen & Schleif, 1977a). Such overproduction has permitted development of techniques which yield up to 1 mg of *araC* protein which is about 20% pure (Steffen & Schleif, 1977a). While these quantities have been sufficient for determination of the binding sites of *araC* protein (Ogden et al., 1980), they are insufficient for study of the means by which *araC* protein recognizes the repression and induction regions on the DNA.

Recently methods have been described for increasing the synthesis of proteins from cloned genes (Guarente et al., 1980; Roberts et al., 1979). Sequences immediately in front of the gene are trimmed away and in their place is fused a sequence containing a highly active promoter and a particularly good ribosome binding site. This approach seemed appropriate to apply to increase synthesis of *E. coli araC* protein. The *araC* promoter in wild-type cells is only about $^1/_{100}$ as active as the derepressed *lac* promoter (Casadaban, 1976), and the translation efficiency of *araC* messenger must be much lower than that of *lac* messenger since the level of *araC* protein, 20 monomers per cell (Kolodrubetz & Schleif, 1981), is far below 1% of the level of derepressed β-galactosidase.

Three findings are presented in this paper. First, removal of some noncoding DNA lying in front of the *araC* gene and the insertion of a DNA fragment containing the *lac* promoter and *lac* ribosome binding site lead to about 5×10^3 greater synthesis of *araC* protein than is found in wild-type cells. Second, this great hypersynthesis is not sensitive to the exact amount of the noncoding DNA preceding the *araC* gene which is removed before insertion of the *lac* fragment, and the hypersynthesis does not require the *lac* ribosome binding site. Third, application of only early steps in previously devised purification procedures (Steffen & Schleif, 1977a) applied to cell extracts containing the elevated levels of *araC* protein yields essentially pure *araC* protein.

Experimental Procedures

Miscellaneous Techniques. Except as noted, enzymes were from New England Biolabs and were used as directed by the supplier. *Bal*31 was from Bethesda Research Labs and was used as directed. T4 polymerase was a gift of William McClure, Harvard University.

DNA sequencing was done by the method of Maxam & Gilbert (1980). Plasmid DNA was cut and labeled at the *Eco*R1 site, and the *Eco*R1-*Hha*I fragment was purified and sequenced.

Constructing the Plasmid Carrying the araC Gene. Approximately 200 ng each of *Bam*H1 cut pRB322 (Bolivar et al., 1977) and λ *para*C116 DNA (Lis & Schleif, 1975) were mixed, ethanol precipitated, and resuspended in 9 μL of 0.05 M Tris-HCl,[1] pH 7.8, and 0.01 M $MgCl_2$. This was incubated 10 min at 65 °C and chilled, and 2 μL of the same buffer but containing 10 mM ATP and 0.1 mM dithiothreitol was added. This was incubated for 2 h at 1 °C with 10 units of Bethesda Research Labs T4 DNA ligase, then 190 μL of the buffer with

† From the Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. *Received May 11, 1981.* Publication No. 1388 of the Department of Biochemistry. This work was supported by U.S. Public Health Service Research Grant GM-18277 from the National Institutes of Health.

[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; IPTG, isopropyl thio-β-D-galactoside; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-0778$01.25/0

ATP and dithiothreitol and 20 units of ligase were added, and the incubation was continued for another 12 h. The DNA was ethanol precipitated, dried, and resuspended in transformation buffer. Approximately 35 ng of DNA yielded 50 Ara^+ transformants of the $AraC^-$ strain DLS 25 on minimal arabinose B1 plates containing 20 μg/mL ampicillin.

Removing One BamH1 Cleavage Site from the araC Plasmids. About 100 μg of DNA from two plasmids each with *araC* inserted in an opposite orientation was digested with *Bam*H1 at a concentration so as to maximize the production of molecules cut at only one of the cleavage sites. Following digestion the opened circles were purified by electrophoresis and extraction from 0.7% agarose. Transformation with this DNA had only 0.1% the infectivity of uncut DNA, and the infectivity was restored to 25% by annealing and ligating prior to transformation.

The sticky ends of the *Bam*H1 sites were filled out with DNA *pol*I by incubation of 5 μg of DNA with 3000 units of enzyme at 18 °C for 1 h in 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 μg/mL bovine serum albumin, and 200 μM in each of the deoxynucleotide triphosphates. The DNA was then phenol extracted, ethanol precipitated, and ligated to closed circular form by the above ligation protocol with DNA at 200 ng/200 μL before transformation and selection of Ara^+ transformants.

Identifying the Plasmid with the Correct Orientation of the araC Insert. The two plasmids containing the *araC* gene insert in opposite orientations and with their single *Bam*H1 sites closer to the *Eco*R1 sites were cut with *Bam*H1 and labeled by filling out the sticky ends with Klenow fragment (Jacobsen et al., 1974) of DNA *pol*I using 100 μm dGTP, dTTP, dCTP, and 2 μM dATP containing 10 μCi of [α-^{32}P]dATP by incubation at 37 °C for 1 h. The DNA was ethanol precipitated and digested with *Hae*III before electrophoresis on 6% acrylamide gel and autoradiography. Similarly labeled *Bam*H1–*Hae*III fragment from the *ara*440 DNA (Smith & Schleif, 1978) provided a size standard.

Fusing the araC Gene to the lac Promoter Ribosome Binding Site. The 95-base-pair *lac* operon fragment containing the promoter and Shine–Dalgarno (1975) ribosome binding region was released from the plasmid pGL101 [similar to pLJ3 (Roberts & Lauer, 1979) but containing only one small *Eco*R1–*Pvu*II fragment] by sequential *Eco*R1 and *Pvu*II digestion and purified by electrophoresis on a 6% acrylamide gel.

Ten micrograms of pRFS11 was cut with *Bam*H1 and digested with exonuclease III and nuclease S1 so as to remove approximately 100 base pairs (Roberts & Lauer, 1979). It was then cut with *Eco*R1. A 10-fold excess of the *lac* 95 fragment and *Bam*H1 linkers was included in the ligation reaction which followed. This DNA was transformed into DLS25 (Steffen & Schleif, 1977a) containing an $F'i^Q$ (Müller-Hill et al., 1968), and candidates were selected in minimal arabinose ampicillin plates.

Acrylamide Gel Electrophoresis. Cells of strain DLS24 (Steffen & Schleif, 1977a) were grown overnight in YT medium, and 0.5 mL of cells was spun down and resuspended in 200 μL of a solution containing 0.15 g of Tris base, 0.4 g of $NaDodSO_4$, 1 mL of 2-mercaptoethanol, 2 mL of glycerol, and 17 mL of H_2O, which was adjusted to pH 6.8 with HCl, and 0.2% bromphenol blue. The sample was heated to 90 °C for 10 min, and 10 μL was loaded on the gels. The gels were 12% acrylamide and 0.72% methylenebis(acrylamide) containing 0.38 M Tris-HCl, pH 8.8, and 0.1% $NaDodSO_4$ and were prepared essentially as described by Weber & Osborn (1969). The stacking gel was 2.5% acrylamide, 0.1% methylenebis(acrylamide), and 0.125 M Tris-HCl, pH 6.8. Gels were run with buffer made from 14.4 g of glycine and 3 g of Tris base in 1 L containing 0.1% $NaDodSO_4$. Gels were stained 1 h in 50% methanol, 10% acetic acid, and 0.2% Coomassie blue and destained several hours in 50% methanol and 10% acetic acid.

Purification and Sequencing araC Protein. araC protein was purified through the phosphocellulose step of the published procedure (Steffen & Schleif, 1977a) and dialyzed extensively against 0.002% $NaDodSO_4$ before lyophilization of 1 mg for sequencing by the Beckman automated Edman sequenator. *araC* protein purified from cells containing pDS1 (Steffen & Schleif, 1977a) through the phosphocellulose step was lyophilized, 5 mg of total protein, and electrophoresed on a 12% acrylamide–$NaDodSO_4$ gel. A strip from this gel was stained to locate *araC* protein, and the remainder of the gel was removed, placed in a dialysis sack, and electrophoretically extracted. The 20 mL of solution was lyophilized, resuspended in 1 mL of 0.002% $NaDodSO_4$, dialyzed against 0.002% $NaDodSO_4$, and lyophilized before sequencing.

Results

Constructing the araC-Containing Plasmid Suitable for Hypersynthesis. The method chosen for increasing synthesis of *araC* protein required that nucleotides in front of the gene be trimmed away with a nuclease and replaced with a DNA fragment containing a good promoter and ribosome binding site. This construction is most convenient with a unique restriction site located just in front of the *araC* gene. Fortunately, a *Bam*H1 cleavage site is located in front of the *araC* gene, and none are located within the gene (Smith & Schleif, 1978; Haggerty & Schleif, 1976). Thus *Bam*H1 cleavage fragments of phage λ p*araC*116 were inserted into *Bam*H1 cleaved plasmid pBR322, and $AraC^+$ transformants were selected. Plasmids from such transformants will possess two, not one, *Bam*H1 cleavage sites, one at each end of the inserted *araC* gene. Also, later steps in the construction necessitate choosing a transformant in which the *araC* gene possesses one of the two possible orientations with respect to the plasmid.

In order to find suitable plasmids, clones were screened by their *Hae*III and *Hha*I restriction digestion patterns to find a pair containing the *araC* gene in opposite orientations. DNA from each pair was then treated to remove one *Bam*H1 cleavage site. This was accomplished by partial digestion with *Bam*H1 so as to cut, on the average, at only one of the sites, filling in the ends with DNA *pol*I and ligating the DNA to re-form circles. DNA isolated from transformants from these two pools of DNA was screened by *Bam*H1 digestion to find plasmids which possessed a single cleavage site. These candidates were then screened by combined *Bam*H1 and *Eco*R1 digestion to find the two, one from each orientation of the C gene, pRFS10 and pRFS11, in which the remaining *Bam*H1 site was on the end of the *araC* gene closer to the *Eco*R1 cleavage site of the plasmid. Finally, the one plasmid from this pair which possessed the *araC* gene in the correct orientation, transcription away from the *Eco*R1 site, was identified by examining the size of the *Bam*H1–*Hha*I fragments from each of these and from a DNA source, *ara*440 (Smith & Schleif, 1978), which provided a control fragment.

Trimming, Fusion, and Selection of the Overproducer. DNA in front of the *araC* gene was trimmed back by digestion with exonuclease III and nuclease S1, and then a 95-base-pair DNA fragment containing the *lac* promoter and ribosome binding site, *lac*95, was fused in. Since the *Bam*H1 site was a long distance from the preseumptive start of the *araC* gene,

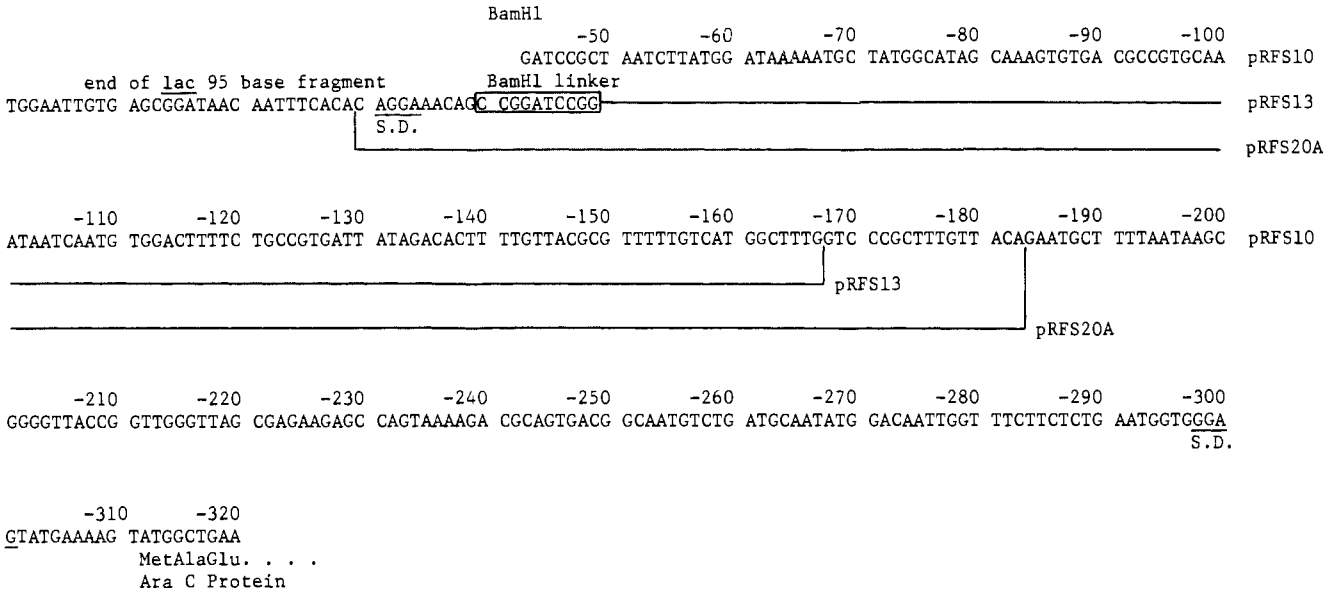

FIGURE 1: DNA sequence of the *ara* regulatory region and the leader region preceding the *araC* gene (Smith & Schleif, 1978; C. Stoner and R. Schleif, unpublished results; Wallace et al., 1980) contained on plasmid pRFS10 as well as the end of the *lac* 95-base-pair fragment containing the promoter and ribosome binding site. The possible ribosome binding sites are underlined and indicated S.D. for Shine & Dalgarno (1975), and the *Bam*H1 linker is enclosed. The nucleotides removed by nuclease digestions in the construction of plasmids pRFS13 and pRFS20A are shown by the lines.

the construction was performed so as to insert a *Bam*H1 linker between the *araC* gene and the *lac* promoter–ribosome binding site DNA fragment. This enabled a second cycle of digestion–fusion to be performed from a much closer distance to the start of the gene.

The possibility that great overproduction of *araC* protein might prove toxic to cells was reduced by cloning into a strain possessing about 10 times the normal amount of *lac* repressor as a result of carrying the *lac* i^Q gene (Müller-Hill et al., 1968). From 10 μg of plasmid which was digested and ligated to the *lac* promoter–ribosome binding site DNA fragment, three candidates transformed to Ara^+ were found (see A Deletion Occurred upon Inserting the *lac* Promoter).

The three candidates were sequenced from their *Bam*H1 sites by the Maxam–Gilbert technique to determine exactly the extent of digestion. One candidate, pRFS13, had the *Bam*H1 site a reasonable distance from the C gene whereas the other two had apparently suffered extensive DNA rearrangements, for the sequence of the DNA next to their *Bam*H1 sites was unrecognizable. It was neither an early portion of the *araC* gene nor a portion of the plasmid vehicle. Between the end of the inserted *Bam*H1 linker of pRFS13 and the initiation AUG of the *araC* gene, 144 base pairs of DNA remained. The digestion had removed 121 base pairs (see Figure 1).

A second cycle of exonuclease III–S1 digestion was performed on pRFS13 to bring the *lac* DNA still closer to the beginning of the *araC* gene. This was done by opening the plasmid with *Bam*H1, digesting with exonuclease III or *Bal*31, removing the remainder of the *lac* 95 fragment by cutting with *Eco*R1, and fusing in an intact *lac* 95 fragment in front of the *araC* gene. This step yielded high numbers, 10^4 Ara^+ transformants per microgram of DNA. Twenty-four of these transformants were purified, and protein extracts of cells were screened on $NaDodSO_4$ gels for possible hypersynthesis of *araC* protein. Cells were grown in the presence and absence of IPTG to identify a protein of the expected molecular weight which was induced when the *lac* promoter was turned on. In fact, most of the candidates appeared to oversynthesize *araC* protein. In the presence of IPTG to induce the *lac* operon, synthesis of *araC* protein increased from 2-fold to 3-fold. The absence of more marked induction indicates that at all times the *lac* promoter in these cells is appreciably induced despite the presence of the i^Q gene to increase repressor levels. The parent plasmid, pRFS13, also appeared to hypersynthesize *araC* protein, although to a somewhat lesser extent. One of these overproducing plasmids was chosen for DNA sequencing, pRFS15, and it proved to have had just the inserted *Bam*H1 linker DNA removed.

Similar constructions were performed by using the double-stranded exonuclease *Bal*31 instead of exonuclease III followed by S1. Transformants were selected independent of whether or not they expressed the *araC* gene, purified, screened genetically for being $AraC^+$, for possessing grossly altered DNA, and for hypersynthesis of *araC* protein. Results similar to the exonuclease III–S1 digestions were obtained. The protein extracts from 12 candidates were examined on $NaDodSO_4$ gels and were found to synthesize *araC* protein to about the same extent as the former candidates. One of these was sequenced, and it left 68 base pairs of the original DNA before the start of the *araC* gene. These results show that the elevated synthesis of *araC* protein is not critically dependent upon the fusion of the *lac* promoter DNA fragment to a particular region in front of the *araC* gene.

Hypersynthesis Does Not Require the lac Ribosome Binding Site. The experiments described above showed that the *araC* protein was synthesized at high levels even though more than 50 bases of the original noncoding material preceding the *araC* gene remained. This result indicates that the *lac* ribosome binding site is unimportant to the *araC* hypersynthesis since gene fusions in which the *lac* ribosome binding site plays an important role function appear to function best with about 10 bases between this site and the initiation AUG (Guarente et al., 1980; Roberts et al., 1979). The experiments described in this section were designed to answer the question of whether or not the ribosome binding site provided by the *lac* DNA is important. It was found not to be. Most likely the *ara* sequence GGAG which is centered 12 bases ahead of the AUG of *araC* functions as an efficient ribosome binding site.

The *lac* ribosome binding site was removed, and the sub-

sequent synthesis of *araC* protein was measured. This was accomplished by opening the plasmid pRFS13 with *Bam*H1, removing about 15 bases in each direction with *Bal*31, and then closing the plasmid. Sequencing the DNA from one transformant, pRFS20A, showed that the digestion had removed 17 bases in the *lac* direction and 21 bases in the *araC* direction, thus removing the AGGA ribosome binding sequence from the *lac* 95 and leaving little resembling a ribosome binding site in its place (Figure 1). The sequence GGAG in front of the *araC* gene remained intact. Gel electrophoresis of the crude extracts prepared from cells containing this plasmid showed them to synthesize *araC* protein to the same extent as their parent, thus demonstrating that the synthesis does not substantially depend upon the presence of the *lac* ribosome binding site.

A Deletion Occurred upon Inserting the lac Promoter. This section deals with an experimental problem which arose during the constructions. Only three transformants were found following the steps to insert the *lac* promoter in front of the *araC* gene, and yet thousands would have been expected if the steps had proceeded with reasonable efficiency. An examination of the structure of the DNA from the resulting plasmid provided a possible explanation for this low number. A sizeable fraction of the plasmid DNA was deleted during the step on inserting the *lac* promoter. Whether creation of such a deletion is a prerequisite to generating a viable *lac–ara* fusion has not been determined, however.

The hypersynthesis plasmid, pRFS13, was shown to be deleted of part of the inserted *E. coli* DNA and part of the plasmid vehicle DNA. Restriction enzymes *Hae*III and *Hha*I were used to digest the parental plasmid, pRFS11, pRFS13, and the original plasmid vehicle, plasmid pBR322. These showed that following the deletion, only about 2000 base pairs of the original 4000-base-pair insert remained. In addition, all the DNA from the *Bam*H1 cleavage site of the plasmid clockwise about 1400 base pairs to somewhere within *Hha*I fragment 3 and *Hae*III fragment 12A on plasmid (Sutcliffe, 1979) was deleted.

Purification of araC Protein. Techniques have been developed for purification of *araC* protein which yield material approximately 20% pure from cells which overproduce *araC* protein about 50-fold (Steffen & Schleif, 1977a). Since the plasmid constructed here leads to the synthesis of *araC* protein to a level about 5000 times at which it is found in wild-type cells, the same purification procedures applied to these cells ought to yield essentially pure *araC* protein. Application of the purification scheme which previously yielded 20% pure *araC* protein now does yield essentially pure *araC* protein, as shown in the third lane of the gel shown in Figure 2. The gel also illustrate the visibility of *araC* protein in crude extracts.

Although the DNA trimming did not approach the coding region of the *araC* gene, it is important to verify that the N terminus of the *araC* protein has not been altered by steps of the construction. Therefore *araC* protein purified from cells containing the hypersynthesis plasmid and *araC* protein purified from the former source, pDS1, were sequenced from the N terminus. The *araC* protein from pDS1 containing cells was purified to 20% purity and then electrophoresed on a preparative $NaDodSO_4$ gel, and the *araC* protein was extracted. These two protein samples yielded the same N-terminal sequence, (Met)-Ala-Glu-Ala-Gln-Asn-Asp-Pro-Leu-Leu....

Discussion

The method chosen here for performing the construction for hypersynthesis of *araC* protein required cloning the *araC* gene into the plasmid pBR322, trimming away some of the material in front of the gene, and replacing this with a 95-base piece of DNA containing the *lac* promoter and *lac* ribosome binding site. The initial constructions were performed as described under Results. However, when the step of inserting the *lac* promoter–ribosome binding site was performed, only three candidates were found instead of the several thousand expected. Examination of the DNA derived from one of these revealed that a portion of the inserted DNA and a portion of the plasmid vehicle DNA had been deleted. The other two had also suffered extensive DNA rearrangements. It is possible that these alterations were necessary to permit viability of the transformants and that the major part of the DNA used in the transformation after the construction was unaltered. It is interesting to note that the region deleted in the plasmid pRFS13, from the insert at the *Bam*H1 site to a site 1400 base pairs clockwise, in *Hae*III fragment 12A, also includes part of a site Twigg & Sherratt (1980) have found to play a role in maintaining copy number of the plasmid.

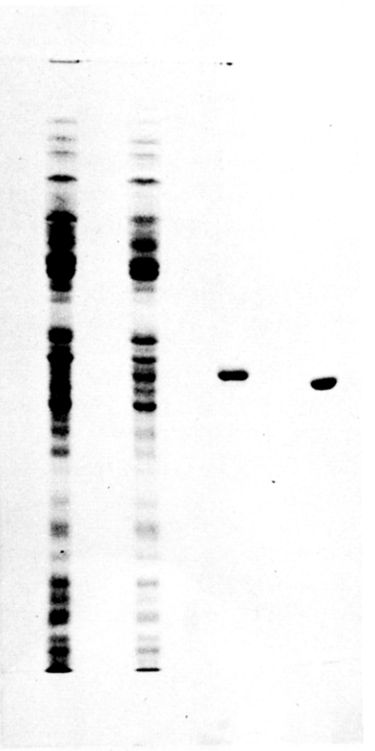

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis pattern of the strain containing the fusion plasmid and the electrophoresis of pure *araC* protein. The first lane shows the electrophoresis pattern of crude extract prepared from strain DLS24 carrying plasmid pBR322. The second lane is a crude extract prepared from the same strain but containing the *araC* overproduction plasmid pRFS15. The *araC* protein encoded by the plasmid is clearly visible. The third lane shows the purified *araC* protein as it elutes from the phosphocellulose column. Faint bands of RNA polymerase are visible in the original gel but are not seen in the photograph. The fourth lane contained carbonic anhydrase as a molecular weight standard.

The *lac* promoter in the plasmids provided a convenient method of adjusting synthesis of the *araC* protein. This facilitated identification of the *araC* protein band on the $NaDodSO_4$ gels.

In contrast to the other fusions which have been constructed to increase synthesis of a protein (Guarente et al., 1980; Roberts et al., 1979), hypersynthesis of *araC* protein was achieved without bringing the *lac* ribosome binding site close to the initiating AUG of the *araC* gene. Apparently a sequence already present in front of the *araC* gene can serve as an efficient ribosome binding site. Thus *araC* synthesis has

increased about 5000-fold above wild-type cells by cloning on plasmid pBR322, by the deletion of some *E. coli* DNA from in front of the *araC* gene, and by the insertion of the *lac* promoter in front of the gene.

Acknowledgments

We thank Tom Roberts for performing the exonuclease III and S1 digestions and Ed Cannon and William Lane for performing the N-terminal sequencing of *araC* protein.

References

Bolivar, F., Rodriquez, R., Greene, P., Betlach, M., Heyneker, H., Boyer, H., Crosa, J., & Falkow, S. (1977) *Gene 2*, 95-113.

Casadaban, M. J. (1976) *J. Mol. Biol. 104*, 557-566.

Englesberg, E., Squires, C., & Meronk, F. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 1100-1107.

Greenblatt, J., & Schleif, R. (1971) *Nature (London), New Biol. 233*, 166-170.

Guarente, L., Lauer, G., Roberts, T., & Ptashne, M. (1980) *Cell (Cambridge, Mass.) 20*, 543-553.

Haggerty, D. M., & Schleif, R. (1976) *J. Virol. 18*, 659-663.

Jacobsen, H., Klenow, H., & Overgaard-Hansen, K. (1974) *Eur. J. Biochem. 45*, 623-627.

Kolodrubetz, D., & Schleif, R. (1981) *J. Mol. Biol. 149*, 133-139.

Lis, J. T., & Schleif, R. (1975) *J. Mol. Biol. 95*, 395-407.

Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 499-560.

Müller-Hill, B., Crapo, L., & Gilbert, W. (1968) *Proc. Natl. Acad. Sci. U.S.A. 59*, 1259-1264.

Ogden, S., Haggerty, D., Stoner, C., Kolodrubetz, D., & Schleif, R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3346-3350.

Roberts, T., & Lauer, G. (1979) *Methods Enzymol. 68*, 473-482.

Roberts, T., Kacich, R., & Ptashne, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 760-764.

Sheppard, D., & Englesberg, E. (1967) *J. Mol. Biol. 25*, 443-454.

Shine, J., & Dalgarno, L. (1975) *Nature (London) 254*, 34-38.

Smith, B. R., & Schleif, R. (1978) *J. Biol. Chem. 253*, 6931-6933.

Steffen, D., & Schleif, R. (1977a) *Mol. Gen. Genet. 157*, 333-339.

Steffen, D., & Schleif, R. (1977b) *Mol. Gen. Genet. 157*, 341-344.

Sutcliffe, J. (1979) *Cold Spring Harbor Symp. Quant. Biol. 43*, 77-90.

Twigg, A., & Sherratt, D. (1980) *Nature (London) 283*, 216-218.

Wallace, R., Lee, N., & Fowler, A. (1980) *Gene 12*, 179-190.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406-4412.

Wilcox, G., Meuris, P., Bass, R., & Englesberg, E. (1974) *J. Biol. Chem. 249*, 2946-2952.

Potent Microtubule Inhibitor Protein from *Dictyostelium discoideum*†

Ted Weinert,‡ Piero Cappuccinelli, and Gerhard Wiche*

ABSTRACT: A novel potent protein factor capable of inhibiting the in vitro polymerization of mammalian brain microtubule protein and of breaking down preformed microtubules has been partially purified from cell extracts of *Dictyostelium discoideum*. The factor has an apparent M_r of around 13 000 and is trypsin resistant but heat and pepsin sensitive. When soluble microtubule protein was fractionated into tubulin and microtubule-associated proteins and each fraction was assayed independently for its susceptibility toward inhibition, it was clearly demonstrated that the tubulin but not the associated protein fraction was rendered nonpolymerizable. Soluble tubulin was inactivated at ratios of 1 mol of inhibitor to 100 mol of tubulin, estimated conservatively. Quantitative separation of tubulin and inhibitor after inactivation did not result in reactivation of tubulin's polymerizing capacity, suggesting a catalytic modification. The biochemical properties tested of the inactive tubulin argue against a mechanism involving simple proteolysis, N-site GTP hydrolysis or release, or general denaturation.

Cytoplasmic microtubules have been shown to play a dynamic role in such diverse motile processes as mitosis, axonal transport, protein secretion, and changes in cell shape (Dustin, 1978). The elucidation of the mechanisms of these events and of the means by which they are regulated depends therefore upon detailed investigations of microtubule components at the molecular level. Since Weisenberg's initial demonstration of the in vitro polymerization of microtubules from mammalian brain (Weisenberg, 1972), much progress has been made in identifying the components and defining their biochemical roles. The two components that have been studied most extensively, guanosine triphosphate and microtubule-associated proteins, are both thought to have positive control functions in the assembly of microtubules, i.e., they facilitate the formation of tubules from soluble tubulin dimers (Kirschner, 1978). Although these factors have been shown to be integral parts of microtubules, evidence suggesting their roles in the dynamic control of microtubule assembly and disassembly in vivo has not been forthcoming.

Regulation by factors acting as negative controls should also exist; they could trigger the disassembly of microtubules and/or maintain large pools on nonpolymerized tubulin in a

† From the Institute of Biochemistry, University of Vienna, 1090 Vienna, Austria (T.W. and G.W.), and the Institute of Microbiology, Medical School, University of Sassari, Sassari, Italy (P.C.). *Received June 1, 1981; revised manuscript received October 30, 1981.* This research was supported by Grants 3353 and 3948 from the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (to G.W.).

‡ Present address: Molecular Biophysics and Biochemistry, Yale University, New Haven, CT.

0006-2960/82/0421-0782$01.25/0