J. Mol. Biol. (1986) **188**, 355–367

Transcription of *Escherichia coli ara in Vitro*

The Cyclic AMP Receptor Protein Requirement for P_{BAD} Induction that Depends on the Presence and Orientation of the *ara*O_2 Site

Steven Hahn†, William Hendrickson‡ and Robert Schleif

Biochemistry Department Brandeis University
Waltham, MA 02254, U.S.A.

(Received 21 September 1984, and in revised form 10 November 1985)

The mechanism by which the cyclic AMP receptor protein, CRP, stimulates transcription of the *Escherichia coli ara*BAD promoter was studied *in vitro*. Under one set of conditions, CRP stimulated by eightfold the rate of RNA polymerase open complex formation on supercoiled DNA template containing the normal wild-type *ara*BAD regulatory region. Since previous studies *in vivo* had identified an upstream site termed *ara*O_2 that is involved in both repression and in the CRP requirement for P_{BAD} induction, we performed similar experiments *in vitro*. Deletion of *ara*O_2 or alterations of its orientation with respect to the *ara*I site by half integral numbers of turns greatly reduced the CRP requirement for induction of P_{BAD}. Linearizing the DNA has the same effect as deleting *ara*O_2 from the supercoiled DNA template. The similarity of conditions that relieve the classical repression of P_{BAD} *in vivo* and the conditions that eliminate the requirement for CRP for maximal activity *in vitro* suggest a close relationship between repression in the *ara* system and the role of CRP.

At lower concentrations of AraC protein and slightly different conditions than those used in the above-mentioned experiments, CRP does stimulate transcription from linear or supercoiled templates lacking *ara*O_2. On linear DNA under these conditions, one dimer of AraC protein binds to linear *ara* P_{BAD} DNA, but is incapable of stimulating transcription without the additional binding of CRP. The responses of the *ara* system under the second set of conditions are unlike its behavior *in vivo*.

1. Introduction

AraC protein regulates initiation of transcription of the *ara*BAD genes and the genes required for the uptake of arabinose (Englesberg *et al.*, 1965; Greenblatt & Schleif, 1971; Wilcox *et al.*, 1974; Kolodrubetz & Schleif, 1981; Kosiba & Schleif, 1982; Stoner & Schleif, 1983). The foremost activity of AraC protein is that of inducing transcription of the *ara* genes in the presence of arabinose. That is, the protein is a positive-acting gene regulator. However, the AraC protein also appears to function in a negative manner (Englesberg *et al.*, 1969*a*). This aspect of its activity is termed repression, even though it now is apparent that the mechanism of repression in *ara*BAD is much different from the repression observed, for example, in the *lac* system (Dunn *et al.*, 1984; Maizels, 1973). Maximal activity of the *ara*BAD promoter, P_{BAD}, also requires the presence of the cyclic AMP receptor protein, CRP, and cyclic AMP (Greenblatt & Schleif, 1971; Lis & Schleif, 1973; Wilcox *et al.*, 1974; Hahn *et al.*, 1984).

The binding site for AraC protein at P_{BAD}, *ara*I, lies between the binding sites for CRP and RNA polymerase (Fig. 1: and see Ogden *et al.*, 1980; Lee *et al.*, 1981). This arrangement suggests a co-operative induction mechanism in which the binding of CRP could assist the binding of AraC protein. Then, the binding of AraC protein to *ara*I could be sufficient to stimulate the binding and/or activation of RNA polymerase from its adjacent binding site.

Data acquired recently, however, do not support the co-operative binding mechanism (Hahn *et al.*,

† Present address: Biology Department 56-409 MIT, Cambridge, MA 02139, U.S.A.

‡ Present address: Department of Microbiology, Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, PE 17033, U.S.A.

0022–2836/86/070355–13 $03.00/0

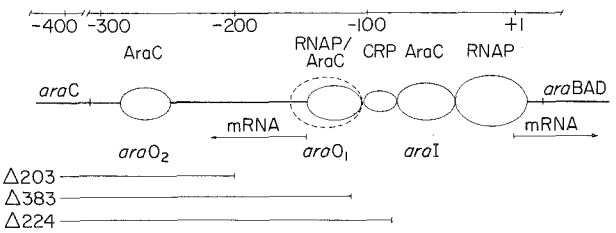

Figure 1. The P_{BAD} regulatory region. The locations of the binding sites for AraC protein, CRP and RNA polymerase are shown. *ara*O_1, *ara*O_2 and *ara*I refer to the P_{BAD} regulatory sites defined by DNase I footprinting and deletion mapping (Ogden *et al.*, 1980; Dunn *et al.*, 1984; Dunn & Schleif, 1984). The endpoints of the deletions used in this study relative to the start of P_{BAD} transcription are: Δ203, −203; Δ383, −126; Δ224, −84. All experiments with the intact P_{BAD} regulatory region contained the DNA sequences from position −404 to +47.

1984; Hendrickson & Schleif, 1984). Instead, the emerging picture for regulation of *ara*BAD suggests that AraC protein binds to the *ara*I site both in the presence and in the absence of arabinose. Upon the addition of arabinose, AraC protein shifts its conformation to a state in which it stimulates transcription from P_{BAD}. While this mechanism explains much data, it raises the question of how CRP stimulates P_{BAD} transcription.

The induction of P_{BAD} appears to be independent of the binding of AraC protein to a second site in the regulatory region, *ara*O_1 (Fig. 1: and see Hahn & Schleif, 1983; Lee *et al.*, 1981). Most likely, the binding of AraC to this site serves to repress its own synthesis from the P_C promoter and is not directly involved in P_{BAD} repression. This aspect of *ara* regulation is not further discussed here.

A third AraC binding site, *ara*O_2, is located 280 base-pairs upstream from the P_{BAD} transcription start site (Fig. 1). This site is required for P_{BAD} repression, as shown in a series of experiments *in vivo* (Dunn *et al.*, 1984). Repression in the *ara* system is manifest in two ways. In the absence of arabinose, the basal level of P_{BAD} increases 5 to 30-fold in cells containing AraC protein but deleted of *ara*O_2 (Englesberg *et al.*, 1969*a*; Dunn *et al.*, 1984). This increase in the basal level likely results from the activity of a small amount of AraC protein that is in the inducing state even in the absence of arabinose. Such a subpopulation of molecules would be capable of stimulating P_{BAD} when *ara*O_2 is absent, but not when it is present.

A second manifestation of repression is revealed by point mutations in P_{BAD}. Ordinarily, in the absence of AraC protein and arabinose, P_{BAD} is barely active *in vivo* and not detectably active *in vitro*. However, some mutations in the RNA polymerase binding site permit some activity from P_{BAD} in the absence of AraC protein (Englesberg *et al.*, 1969*b*; Hahn *et al.*, 1984). The introduction of AraC protein without the addition of arabinose decreases activity from these mutant promoters. This repression does not occur if *ara*O_2 is deleted. Also, a mutation in the *ara*I site can eliminate this repression (Hahn *et al.*, 1984). These results indicate that AraC protein in its non-inducing state acts in association with both the *ara*O_2 and *ara*I sites to repress the mutant P_{BAD} promoters.

To explain the data described above, we propose as a working hypothesis that repression of P_{BAD} occurs by AraC binding to both *ara*O_2 and *ara*I, and that these two proteins bind one another. This would form a DNA loop. By this hypothesis, the loop structure helps hold the protein in a non-inducing state (Dunn *et al.*, 1984; Hahn *et al.*, 1984), and the addition of arabinose alters the conformation of AraC protein at *ara*I, perhaps by opening the loop. Evidence for the looping mechanism is that alteration of either of these two AraC binding sites, whose centers are separated by 210 base-pairs, interferes with repression. Also, introducing spacing mutations between *ara*O_2 and *ara*I that change the angular orientation of these two sites with respect to one another by half integral turns of the DNA could make formation of the loop difficult and have been found to interfere with repression (Dunn *et al.*, 1984). Spacing changes that generate integral numbers of turns presumably return the proteins to their original orientations and appear not to interfere with repression. The presence of two widely separated sites, both required for repression of *Ara*BAD, is similar to the discovery of two essential operator sites in the *gal* operon separated by 110 base-pairs (Irani *et al.*, 1983).

The mechanism by which CRP stimulates transcription from promoters is not at all clear. The protein binds to promoters in different positions with respect to the transcription start site, and the protein appears to stimulate transcription of different promoters by different mechanisms (de Crumbrugghe *et al.*, 1984; Yu & Reznikoff, 1984; Malan & McClure, 1984). Recent work has shown a connection between the *ara*O_2 site and induction of P_{BAD}. Deletion of the *ara*O_2 site greatly reduces the requirement for CRP in the induction of P_{BAD} *in vivo* (Hahn *et al.*, 1984).

Here we describe our experiments *in vitro* examining the role of CRP in the induction of *ara* P_{BAD}. Our experiments lead to the conclusion that CRP can function on *ara*BAD *in vitro via* two modes. One appears to disrupt the effect of the DNA loop between *ara*O_2 and *ara*I, and requires supercoiled DNA, and the other does not require *ara*O_2 and appears independent of supercoiling.

2. Materials and Methods

(a) *General methods and materials*

All general procedures such as transformations, plasmid DNA isolation, cloning, and electrophoresis were as described by either Schleif & Wensink (1981) or Maniatis *et al.* (1982). All restriction enzymes were obtained from either New England Biolabs, Boehringer-Mannheim, Bethesda Research Labs, or PL Biochemicals.

Nucleotide triphosphates were from PL Biochemicals and [α-^{32}P]UTP was from New England Nuclear. All other chemicals were obtained from either Sigma Chemical Co., Fisher Scientific, or Bio-Rad Laboratories.

(b) *Proteins*

AraC protein was purified from cells containing the AraC protein overproducing plasmid pRFS13 (Schleif & Favreau, 1982), through the phosphocellulose column step as described by Steffen & Schleif (1977). The protein was greater than 90% pure as judged by polyacrylamide gel electrophoresis and was about 25% active in specific DNA binding (Hendrickson & Schleif, 1984). Samples of AraC protein stored at -70°C in buffer containing 50% (v/v) glycerol were completely stable. When thawed and stored at -20°C, the protein lost less than 30% activity in 2 to 4 weeks.

CRP was purified by the method of Eilen *et al.* (1978) from strain BS646 that overproduces CRP approximately 50-fold (Cossart & Gicquel-Sanzy, 1982). The CRP was greater than 90% pure and was about 25% active in specific DNA binding (Hendrickson & Schleif, 1984). RNA polymerase was purified from strain PR7 (Reiner, 1969) by the procedure of Burgess & Jendrisak (1975) as modified by Lowe *et al.* (1979). The RNA polymerase was greater than 90% pure and was about 50% active in specific binding to the *ara*C promoter, P_C, as measured by the gel electrophoresis DNA binding assay (Hendrickson & Schleif, 1984). Molar concentrations given for all proteins refer to the amount of protein active in specific DNA binding as measured by the gel electrophoresis DNA binding assay.

(c) *DNA templates for transcription* in vitro

All templates used for transcription *in vitro* were derivatives of the *ara-gal*K fusion plasmid pTD3 (Dunn *et al.*, 1984). These plasmids contained either the intact P_{BAD} regulatory region from position -404 to $+47$ or the deletions indicated in Fig. 1. To compare transcription from both linear and supercoiled DNA templates, the *rpo*C terminator for transcription (Squires *et al.*, 1981) was cloned downstream from P_{BAD} at position $+50$. The *rpo*C terminator was isolated from plasmid pAD9 (provided by C. Yanofsky) on an approx. 240 base-pair *Eco*RI fragment. The fragment was made blunt-ended by filling out the sticky ends with DNA polymerase and was then ligated into the *Hin*dIII site of the *ara-gal*K fusion plasmids, which also had been filled out with DNA polymerase. From these templates, initiation of transcription at P_{BAD} produces an approx. 170-nucleotide transcript terminating at the *rpo*C terminator. *In vitro*, termination of transcription at the *rpo*C terminator was greater than 90% efficient.

For transcription experiments using supercoiled DNA, the plasmids were purified by CsCl/ethidium bromide density-gradient centrifugation followed by chromatography on Biogel A15. The plasmids were stored in TE buffer (10 mM-Tris · HCl (pH 8), 1 mM-EDTA) at 4°C and used directly in the transcription reactions. Typical DNA preparations contained about 80% supercoiled and 20% relaxed DNA. For transcription with linear DNA, the supercoiled plasmids were digested with *Sma*I, which cuts at a unique site about 17 base-pairs downstream from the cloned *rpo*C terminator fragment. Following digestion, the DNA samples were heated to 65°C for 15 min, precipitated in ethanol twice and stored at 4°C in TE buffer. DNA concentrations were measured both spectrophotometrically and by agarose gel electrophoresis followed by staining with ethidium bromide and comparison against appropriate standards.

(d) *Transcription* in vitro

Transcription was performed *in vitro* by a modification of the procedure described by Hirsh & Schleif (1977) and Brown (1979). CRP and AraC protein were added to 20 μl of 0·25 to 0·5 nM-DNA template in 20 mM-Tris · HCl, 100 mM-KCl, 10 mM-$MgCl_2$, 0·5 mM-EDTA, 1 mM-dithioerythritol, 50 mM-L-arabinose, and 50 μg bovine serum albumin/ml at the indicated temperature and pH. The proteins were allowed to bind the DNA template for 5 to 10 min. RNA polymerase was then added and, at the indicated times later, heparin and nucleoside triphosphates were added to final concentrations of 100 μg heparin/ml, 200 μM-ATP, GTP, 100 μM-CTP and 10 μM-UTP (15 to 7·5 μCi/nmol [α-^{32}P]UTP). RNA polymerase was allowed to elongate RNA transcripts at 37°C for 10 to 15 min. The reactions were stopped by addition of 20 μl of 85% (v/v) formamide, 40 mM-EDTA, 0·05% (w/v) bromphenol blue and xylene cyanol. Samples were heated to 65°C for 4 min, chilled on ice, and analyzed by electrophoresis on 5% (w/v) acrylamide/7·5 M-urea gels followed by autoradiography. The amount of specific RNA transcripts made was quantified by excising the RNA band from the gel and counting directly using the Čerenkov effect. In the *in vitro* transcription system used here, P_{BAD} expression was stimulated greater than 20-fold by both AraC protein and arabinose, as expected. Addition of the anti-inducer D-fucose inhibited induction by arabinose, as has been reported both *in vivo* and *in vitro* (Englesberg *et al.*, 1965; Greenblatt & Schleif, 1971; Lee *et al.*, 1974; Wilcox *et al.*, 1974; Hirsh & Schleif, 1977).

The intact plasmids used as templates for transcription *in vitro* served as templates for a number of other RNAs in addition to P_{BAD}. As shown in Fig. 2, this includes both high molecular weight RNA and a short 110-nucleotide transcript made from the RNA-I promoter at the origin of plasmid DNA replication (Levine & Rupp, 1978; Morita & Oka, 1979; Rosenberg *et al.*, 1983).

Taking into account the sequence of the RNA transcript, the specific activity of the UTP, and the efficiency of Čerenkov counting in the polyacrylamide gel slices, the fraction of DNA molecules transcribed under fully induced conditions was calculated for both the linear and supercoiled DNA templates. The supercoiled template was very efficient for P_{BAD} transcription with 90($\pm$10)% of the supercoiled DNA molecules producing an RNA transcript. However, the utilization of the linear template was much less with only 15($\pm$5)% of the DNA molecules producing an RNA transcript. Increasing the level of RNA polymerase or increasing the time of RNA polymerase association did not increase the utilization of the linear template. Transcription of the linear DNA template was also much more temperature-sensitive than that from supercoiled DNA. Under our conditions, the rate of RNA polymerase association and the fraction of templates utilized on supercoiled DNA were nearly constant between 37 and 31°C but both were reduced about 2-fold at 32°C on linear DNA.

(e) *Gel electrophoresis DNA binding assay*

The gel binding assay was performed essentially as described by Hendrickson & Schleif (1984). This assay

was done in parallel with the transcription reaction *in vitro* using the same DNA template, buffer and proteins as used for transcription reactions. To monitor binding of AraC protein to *ara*I, 10^{-14} M-^{32}P-labeled Δ383 DNA (Fig. 1: Hendrickson & Schleif, 1984) was added to the DNA binding reaction. This 167 base-pair DNA fragment contains only the CRP, *ara*I and P_{BAD} RNA polymerase binding sites. CRP and AraC protein were allowed to bind the DNA template for 10 min at 37°C. Glycerol was added to 5% and the reactions were immediately loaded onto a 6% polyacrylamide gel to separate protein–DNA complexes from free DNA. DNA binding was visualized by autoradiography and quantified by densitometry.

(f) *DNase I footprinting*

DNase I footprinting was performed essentially as described by Ogden *et al.* (1980). Footprinting was performed under conditions identical to those of the transcription and gel binding assays using identical DNA, buffers and proteins. In order to visualize the DNase footprinting by autoradiography and to maintain nearly the same level of both specific and non-specific DNA as in the transcription and gel binding assays, the footprinting reactions contained equal molar amounts of linear plasmid pΔ383T and (γ-^{32}P)-labeled Δ383 fragment at a specific activity of 3×10^7 cts/min per μg of fragment. CRP and AraC were allowed to bind the DNA template for 10 min at 37°C and DNase I was added to a final concentration of 1·5 μg/ml for 15 s. The reactions were stopped by the addition of 20 μl of 4·5 M-ammonium acetate, 0·25 μg tRNA/ml and precipitated in ethanol. Samples were analyzed by electrophoresis followed by autoradiography.

3. Results

(a) *CRP stimulation at high levels of AraC protein*

(i) *CRP requirements dependent upon* araO_2

To study *ara*BAD transcription *in vitro* under conditions as close as possible to the situation *in vivo*, we wished to be able to use supercoiled DNA templates and to observe short transcripts that could be separated from other transcripts by gel electrophoresis. Therefore, we constructed a series of supercoiled DNA templates with the *rpo*C terminator for transcription (Squires *et al.*, 1981) inserted downstream from P_{BAD} (see Materials and Methods). From these templates, transcription initiating at P_{BAD} produces a 170-nucleotide transcript that terminates at the *rpo*C terminator.

In most experiments, the activity of P_{BAD} was assessed by measuring the rate of RNA polymerase open complex formation. This was done by simultaneously blocking further open complex formation by the addition of heparin to reaction mixtures, and permitting the open complexes to transcribe by providing nucleoside triphosphates. The amount of open complex present was taken to be proportional to the amount of transcript synthesized.

Transcription conditions were sought in which the CRP responses of P_{BAD} *in vitro* paralleled the responses *in vivo*. On the wild-type P_{BAD} promoter *in vivo*, less than 5% induction is observed in the absence of CRP, even when AraC protein is present at 20 times its normal level (Hahn *et al.*, 1984). However, when the *ara*O_2 site is deleted from P_{BAD}, the promoter can be stimulated to at least 50% of its normal activity in the absence of CRP. Therefore, in addition to the requirement that P_{BAD} be induced by the presence of AraC protein and arabinose, the criterion of a faithful transcription system *in vitro* was that CRP be required for transcription and provide a significant stimulation only when *ara*O_2 was present on the template.

Figure 2 shows that, under the conditions noted, P_{BAD} contained on a supercoiled template possesses the expected properties. The rate of open complex formation on wild-type *ara* P_{BAD} in the presence of AraC protein and arabinose is eightfold greater in the presence of CRP than in its absence. However, with *ara*O_2 deleted, only a slight CRP stimulation is observed in the presence of AraC protein and arabinose. This slight stimulation is reproducible and will be addressed in a later section. The rate of open complex formation on supercoiled Δ*ara*O_2 template in the absence of CRP nearly equals the rate on wild-type DNA (*ara*O_2 present) with CRP present.

Under the conditions used in the experiment shown in Figure 2, levels of both AraC and CRP were saturating, since increasing the levels of either protein fourfold did not increase the level of transcription or the rate of open complex formation. Additionally, increasing the level of RNA polymerase twofold did not detectably increase the rate of open complex formation on either DNA template. However, repeating the experiment of Figure 2 under different conditions showed that the response was sensitive to temperature and ionic strength (Fig. 3). Increasing the temperature from 32°C to 37°C or decreasing the salt and Mg^{2+} levels increased the rate of open complex formation from the wild-type DNA template in the absence of CRP. In separate experiments with supercoiled DNA template lacking *ara*O_2 with or without CRP present, we found that the activity of P_{BAD} increases by less than 10% as the temperature is increased from 32°C to 37°C. These results show that the major effect of the temperature is not upon the intrinsic activity of RNA polymerase on P_{BAD} when it is present on supercoiled template, but upon the ability of *ara*O_2 to inhibit the rate of open complex formation. Therefore, to facilitate study of this effect, we performed most experiments with supercoiled DNA at 32°C. Unfortunately, although the fraction of DNA templates utilized with supercoiled templates is near 100% at 37°C, it is only 20% on linear DNA, and this falls to about 10% as the temperature is decreased to 32°C. Due to such a low activity at 32°C, most of the experiments with linear DNA described in section (b), below, had to be performed at 37°C.

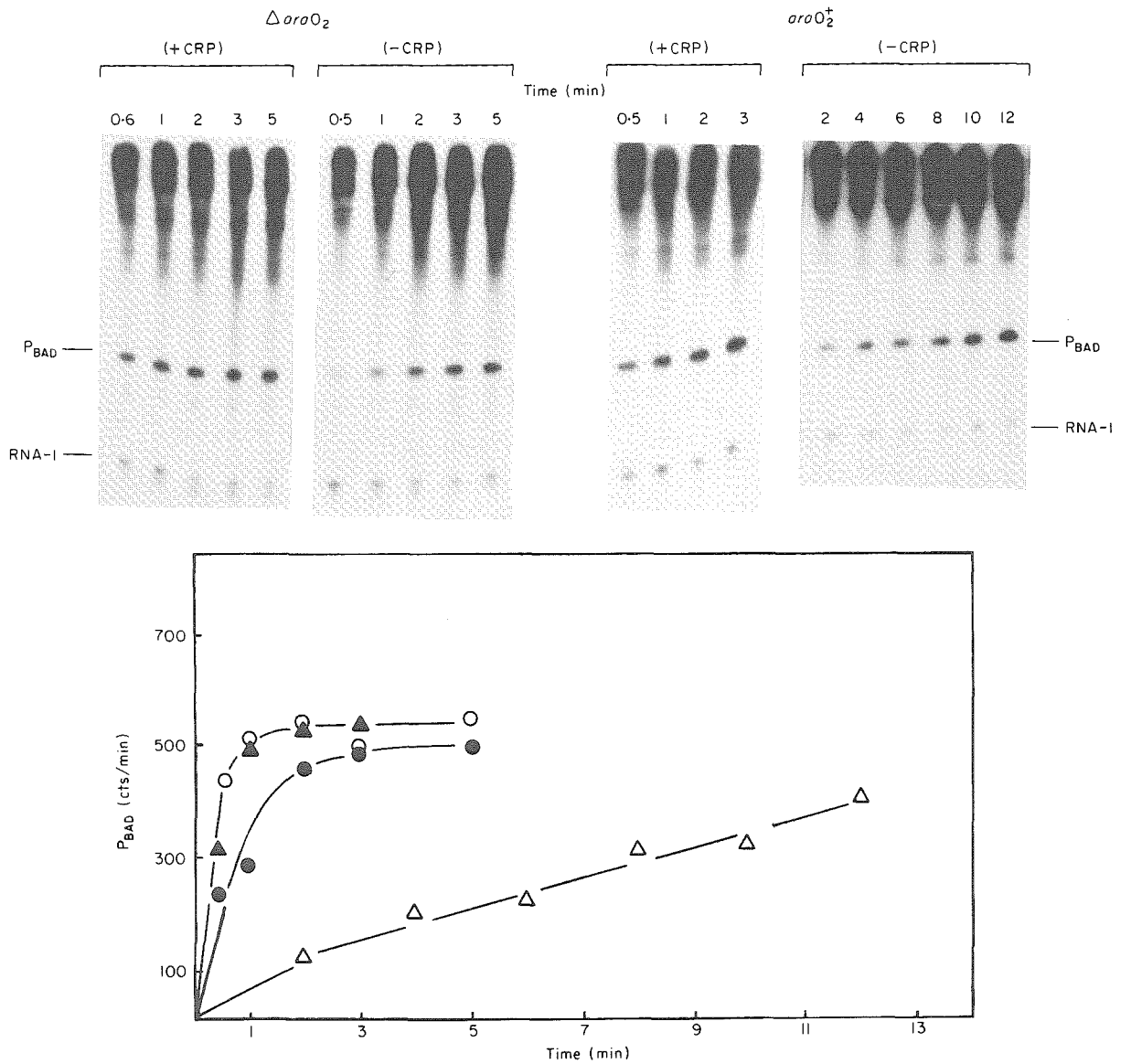

Figure 2. Top: Transcription performed *in vitro* with the supercoiled plasmids pΔ203T deleted for *ara*O_2 and p3T containing the intact P_{BAD} regulatory region with or without 5 nM-CRP. Shown are the RNA transcripts originating from both the P_{BAD} and RNA-1 promoters. Bottom: (●) Δ*ara*O_2, −CRP; (○) Δ*ara*O_2, +CRP; (△) *ara*O_2^+, −CRP; (▲) *ara*O_2^+, +CRP. The amounts of specific P_{BAD} transcripts were quantified and are plotted *versus* the time of RNA polymerase association before heparin addition: 1·5 nM-AraC protein and CRP were pre-incubated with 0·25 nM-DNA template for 10 min and, at time zero, 5 nM-RNA polymerase was added. At the indicated times later, heparin and nucleotide triphosphates were added to stop RNA polymerase association. Protein binding was performed at 32°C, pH 7·5 in the standard buffer described in Materials and Methods: 1 nmol of RNA product corresponds to $2{\cdot}3 \times 10^3$ cts/min.

(ii) *Altering the distance or angular orientation between* araO_2 *and* ara*I alters the CRP requirements of induction*

Experiments *in vivo* have shown that deletion of *ara*O_2 eliminates repression of P_{BAD} and greatly reduces its CRP requirement for induction. In addition, the introduction of spacing mutations between *ara*O_2 and *ara*I that alter the angular orientation of these two sites with respect to one another by half integral turns of the DNA helix interferes with repression of P_{BAD}. Our experiments *in vitro* described above showed that deletion of the *ara*O_2 site eliminates the CRP requirement for P_{BAD} induction, just as it does *in vivo*. We therefore asked whether changing the angular orientation between *ara*O_2 and *ara*I alters the CRP requirements of P_{BAD} *in vitro*.

Spacing mutations generated by insertions of five or 11 base-pairs, and a deletion of eight base-pairs, all made at position −203, were tested. In this experiment, done in the absence of CRP, the rate of open complex formation on wild-type supercoiled

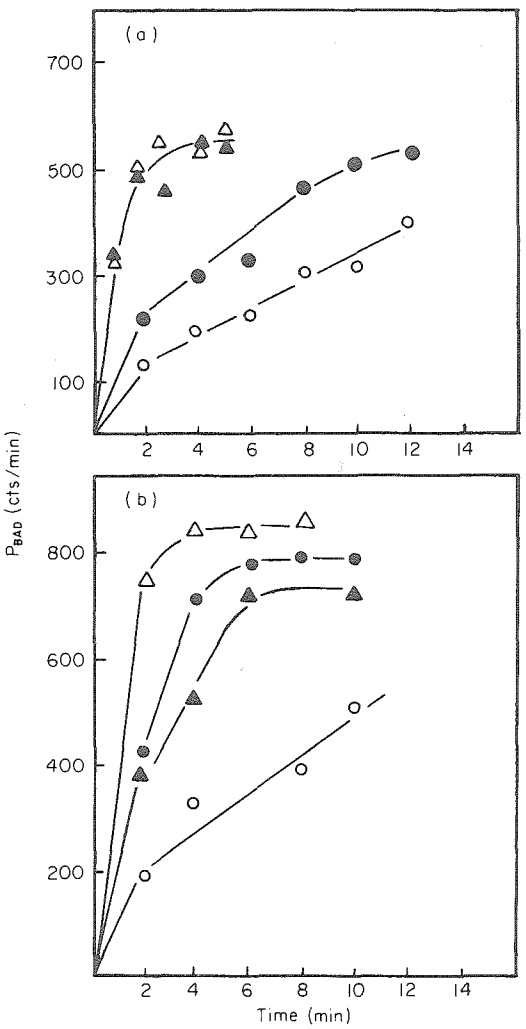

Figure 3. *ara*O_2^+ transcription is sensitive to both temperature and ionic strength. (a) (○) O_2^+, 32°C; (△) ΔO_2, 32°C; (●) O_2^+, 37°C; (▲) ΔO_2, 37°C, all at 100 mM-KCl, 10 mM-$MgCl_2$. (b) (○) 100 mM-KCl, 10 mM-$MgCl_2$; (▲) 50 mM-KCl, 10 mM-$MgCl_2$; (●) 100 mM-KCl, 5 mM-$MgCl_2$; (△) 100 mM-KCl, 2·5 mM-$MgCl_2$. Transcription *in vitro* performed in the absence of CRP from supercoiled plasmid containing the intact P_{BAD} regulatory region in the transcription buffer; (a) pH 7·4 with template *ara*O_2 as indicated; (b) pH 7·5 with *ara*O_2^+ template. Plotted are the amount of P_{BAD} transcripts (P_{BAD}, cts/min) *versus* the time of RNA polymerase association before heparin addition. AraC protein (1·5 nM) was pre-incubated with 0·25 nM-DNA template for 10 min. At time zero, 5 nM-RNA polymerase was added and, at the indicated times later, heparin and nucleotide triphosphates were added. In (a) 1 nmol of RNA product corresponds to $2{\cdot}6 \times 10^3$ cts/min; and in (b) 1 nmol corresponds to $3{\cdot}7 \times 10^3$ cts/min.

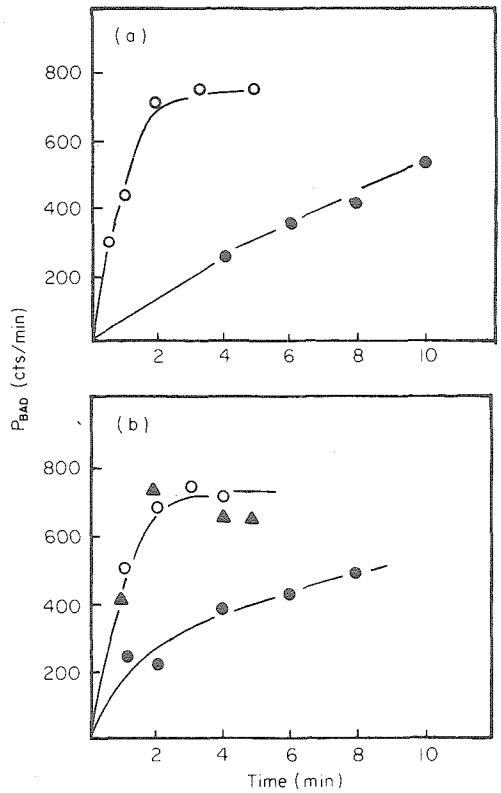

Figure 4. DNA with an insertion or a deletion generating a change of less than an integral number of helical turns between *ara*O_2 and *ara*I behaves similar to DNA lacking the *ara*O_2 site. (a) (○) Δ*ara*O_2, −CRP; (●) wild-type, −CRP; (b) (○) +5 base-pair insertion, −CRP; (▲) −8 base-pair deletion, −CRP; (●) +11 base-pair insertion, −CRP. Transcription *in vitro* using supercoiled plasmid DNAs. Plotted are the amounts of P_{BAD}-specific RNA transcripts (P_{BAD}, cts/min) *versus* the time of RNA polymerase association before heparin addition. AraC protein (1·5 nM) was pre-incubated for 10 min with 0·25 nM-DNA template at 32°C. At time zero, 5 nM-RNA polymerase was added and, at the indicated times later, heparin and nucleotide triphosphates were added: 1 nmol of RNA product corresponds to $3{\cdot}7 \times 10^3$ cts/min.

P_{BAD} template was 20% the rate of open complex formation in the absence of *ara*O_2 (Fig. 4(a)), thus repeating the result that deletion of *ara*O_2 greatly reduces the CRP requirement for induction of P_{BAD}.

With the DNA templates containing the five base-pair insertion or eight base-pair deletion, the rate of open complex formation in the absence of CRP was nearly the same as the rate on *ara*O_2-deleted DNA (Fig. 4(b)). This shows that rotation of the *ara*O_2 site by about half a helical turn is equivalent to deleting it. However, on the DNA template containing the 11 base-pair insertion, the rate of open complex formation in the absence of CRP was reduced to a rate nearly equal to that of the wild-type template in the absence of CRP, thus showing that introduction of a full turn restores wild-type behavior. In the presence of CRP, the rate of open complex formation with the +11 template equalled that of wild-type DNA and the rates with the −8 and +5 templates doubled, as does the rate on template deleted of *ara*O_2 (not shown). This final result shows that CRP has an effect in addition to that of inhibiting the activity of *ara*O_2 (also see Fig. 2).

These results were obtained *in vitro* with a transcription system containing only supercoiled DNA, RNA polymerase, CRP and AraC protein.

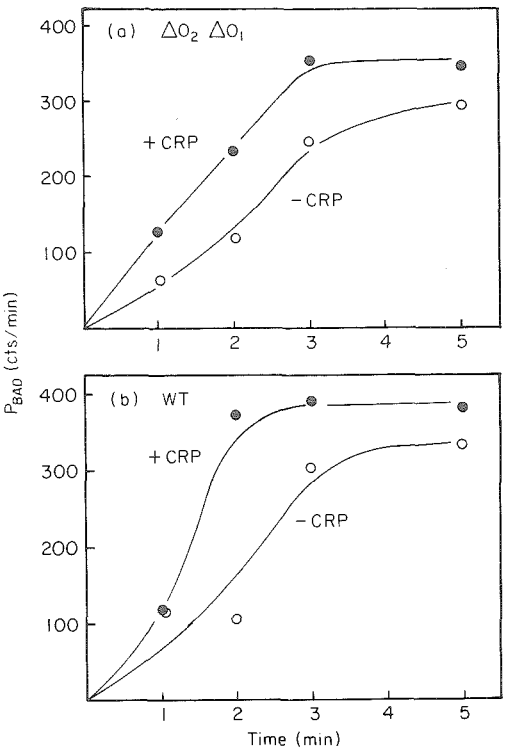

Figure 5. Transcription from linear DNA is insensitive to the presence of *ara*O_2. (a) (●) Δ*ara*O_2, Δ*ara*O_1, +CRP; (○) Δ*ara*O_2, Δ*ara*O_1, −CRP; (b) (●) *ara*O_2^+, *ara*O_1^+, +CRP; (○) *ara*O_2^+ *ara*O_1^+, −CRP. DNAs were pΔ383 or p3T. Transcription was carried out with or without 5 nM-CRP. Plotted is the amount of P_{BAD} specific RNA transcript (P_{BAD}, cts/min) *versus* the time of RNA polymerase association before heparin addition. AraC protein (2 nM) and CRP, when present, were pre-incubated with 0·5 nM-DNA template for 10 min. At time zero, 10 nM-RNA polymerase was added and, at the indicated times, heparin and nucleotide triphosphates were added. Protein binding was performed in standard transcription buffer, pH 7·4, 37°C: 1 nmol of RNA product corresponds to 5×10^3 cts/min.

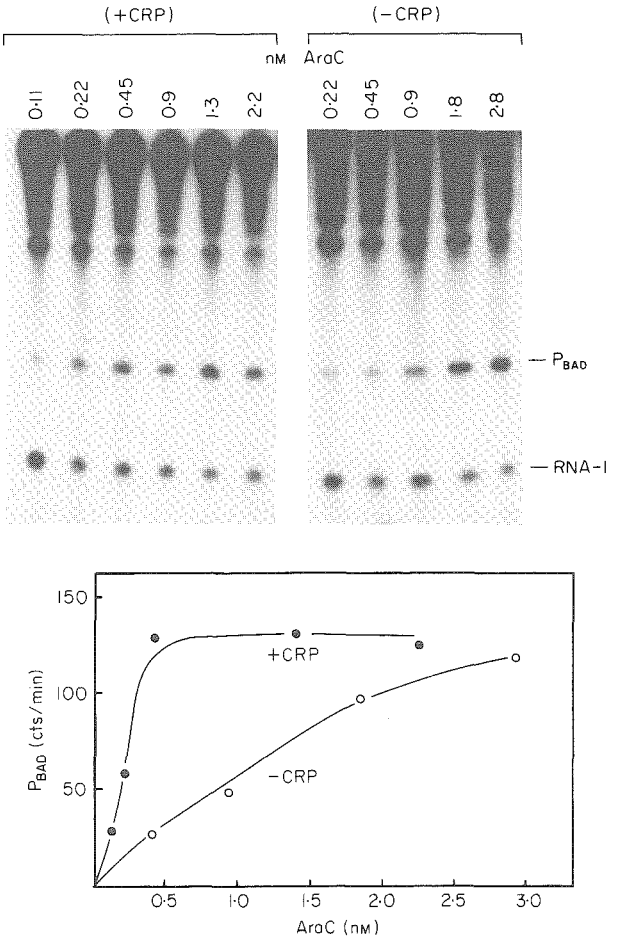

Figure 6. At low levels of AraC protein, CRP can stimulate P_{BAD} transcription on a linear template deleted of *ara*O_2. Top: Transcription *in vitro* at various levels of AraC protein was performed from the linear plasmid pΔ383T deleted for both *ara*O_1 and *ara*O_2. The RNA transcripts initiating from both P_{BAD} and the RNA-1 promoters are indicated. Bottom: (●) Δ*ara*O_2, Δ*ara*O_1, +CRP; (○) Δ*ara*O_2, Δ*ara*O_1, −CRP. The amount of specific P_{BAD} transcript was quantified and is plotted *versus* the level of AraC protein added. Transcription was performed with or without 2·5 nM-CRP. AraC and CRP were pre-incubated with 0·3 nM-DNA template for 10 min followed by the addition of 10 nM-RNA polymerase. After 10 min, heparin and nucleotide triphosphates were added. Transcription was performed under standard buffer conditions, pH 7·8, 37°C: 1 nmol of RNA product corresponds to $2{\cdot}8 \times 10^3$ cts/min.

The fact that nothing else was required to reproduce the requirements for CRP *in vivo* suggests that the same system likely does not require other unknown components *in vivo*.

(iii) *Supercoiling and high AraC concentrations may be required for the O_2-dependent CRP requirement*

Changing from supercoiled to linear DNA has the same effect as deleting *ara*O_2 (Fig. 5). Furthermore, the 20 to 50% CRP stimulation that is observed is a secondary effect of CRP, which will be discussed below. One interpretation of the requirement for supercoiled DNA is that supercoiling assists loop formation *in vitro* and that the loop does not readily form with linear DNA. Therefore, the CRP requirement of P_{BAD} is largely alleviated with linear template. Why then have some previous studies (Lee *et al.*, 1974; Hirsh & Schleif, 1977) reported a substantial CRP stimulation of P_{BAD} activity using linear templates?

At lower pH values and at concentrations of AraC protein below 1·5 nM we do observe a significant stimulation by CRP (Fig. 6). An approximately threefold stimulation in the fraction of template utilized occurs on linear (Fig. 6) or supercoiled DNA (not shown) at 0·45 nM-AraC at pH 7·8. With both linear and supercoiled templates, the CRP stimulation is independent of the presence of *ara*O_2.

Up to the experiment of Figure 6, all experiments presented were done at pH 7·4 or 7·5. The experiment presented in Figure 6 and all

subsequent experiments were performed at pH 7·8. Although the CRP stimulation effect as displayed in Figure 6 is present when the experiment is performed at pH 7·4 to 7·5, it is larger and therefore easier to examine when the experiment is done at pH 7·8. As a result of the difference between the conditions used in the two series of experiments, comparisons have not been made. Whether the phenomenon under investigation in the second series of experiments has physiological significance remains to be determined.

(b) *Studies with linear DNA*

(i) *Binding of one molecule of AraC is not sufficient to activate P_{BAD} on linear templates*

How does CRP accelerate the rate of open complex formation on P_{BAD} when AraC protein is present at low concentrations? This question was approached by comparing transcription and DNA binding by the proteins as discussed in the following two sections. The gel electrophoresis assay is a convenient method for monitoring specific binding by AraC protein and CRP (Hendrickson & Schleif, 1984). Since this assay requires the use of linear DNA molecules, we added small quantities of labeled 167 base-pair linear fragment containing only the CRP, *ara*I and RNA polymerase sites to larger quantities of linearized plasmid DNA in the parallel transcription and binding experiments described below (see Materials and Methods).

Figure 7 shows the gel from an assay of CRP and AraC protein binding to the 167 base-pair fragment. This Figure shows the assay results plotted as the amount of DNA in the AraC–DNA complexes as a function of the concentration of AraC protein in the

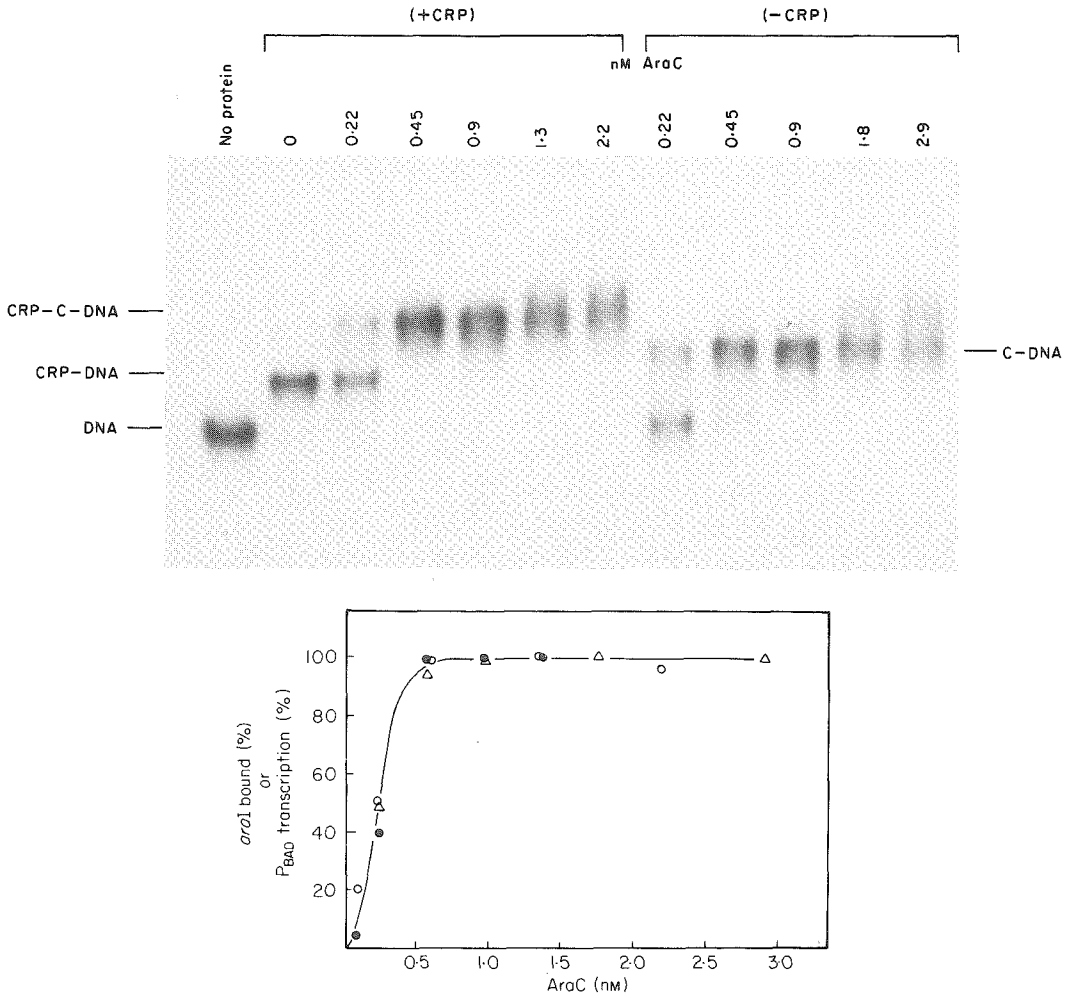

Figure 7. Parallel gel electrophoresis DNA binding assay and transcription *in vitro*. Top: Gel binding assay done in parallel with the transcription experiment of Fig. 6. The reactions contained 0·3 nM-linear Δ383T DNA and 10^{-5} nM-(γ-^{32}P)-labeled Δ383 fragment under identical buffer conditions to those for Fig. 6. AraC protein with or without 2·5 nM-CRP were incubated with the DNA for 10 min at 37°C before separating protein–DNA complexes from free DNA by electrophoresis. The positions of free DNA, AraC–DNA, CRP–DNA, and AraC–CRP–DNA complexes are indicated. Bottom: The amount of protein DNA complex was quantified by densitometry and is plotted as the percentage of *ara*I site bound either with (●) or without (△) CRP added. (○) Indicates the percentage of maximal P_{BAD} transcription in the presence of CRP from the experiment of Fig. 6. The additional binding of AraC protein to DNA above 1·8 nM in the gel binding assay probably represents non-specific binding and is not related to the stimulation of transcription in the absence of CRP.

reaction. This experiment was performed in parallel with the experiment shown in Figure 6, except for omission of RNA polymerase from the samples assayed for AraC protein binding. Therefore the number of AraC–DNA complexes may be compared directly with the number of open complexes formed.

The results show no co-operativity in the binding of CRP and AraC protein to the DNA, as reported previously (Hendrickson & Schleif, 1984). More importantly, they show that when CRP is bound, transcription is proportional to the amount of AraC protein bound (compare filled and open circles in Fig. 7). However, when CRP is absent, there is binding by AraC protein, but little formation of open complexes (compare open triangles in Fig. 7 to open circles in Fig. 6). These results obtained with 0·45 nM-AraC protein agree with the previous observation (Hendrickson & Schleif, 1984) that, when CRP was absent, the binding of one molecule of AraC–arabinose at *ara*I was insufficient to stimulate RNA polymerase binding at P_{BAD}. Increasing the concentration of AraC protein still more does activate P_{BAD} (Fig. 6), thus indicating that an additional molecule of AraC protein is required for induction of P_{BAD} in the absence of CRP under these conditions.

One possible cause of an artifact in the results discussed above is the presence of a low-level contaminant in the AraC protein preparation, CRP for example, such that at high concentrations of AraC the contaminant provides the CRP function. This seems unlikely in view of the requirement for CRP when high levels of AraC protein are used with supercoiled DNA templates containing *ara*O_2. Another possible complexity in these experiments could be interactions by AraC or RNA polymerase at sites upstream from *ara*I. This possibility was excluded by the finding that template deleted of such sequences, pΔ224 (Fig. 1), yielded data identical to that shown in Figure 6, in which CRP was omitted. The deletion did not interfere with the ability of the higher concentrations of AraC to stimulate P_{BAD} transcription.

(ii) *AraC protein stimulates transcription in two ways*

The experiments of the previous two sections have shown that at low concentrations of AraC protein (0·45 nM) all the DNA molecules in the reactions contained a bound AraC molecule, and stoichiometry measurements (Hendrickson & Schleif, 1985) show that the bound species is a dimer of the protein. However, increasing the concentration of the protein to 3 nM permitted transcription to occur. Does an additional dimer of AraC protein bind to the DNA or the protein–DNA complex? The gel binding assay as shown in Figure 7 does not give a clear indication of additional binding, for a second dimer of AraC could bind, but with an affinity too low to remain on the complex during the electrophoresis.

DNase I footprinting is one method of looking for additional binding at the higher concentrations of AraC protein. It was possible to perform the DNase footprinting under conditions identical to those used in the gel binding portion of the experiment shown in Figure 7. Surprisingly, at a level of AraC protein–arabinose that showed complete binding in the gel binding assay, 0·45 nM, no protection of *ara*I was observed (Fig. 8). Under these conditions, only a weakly enhanced cleavage at position −60 appeared. Increasing the level of AraC protein to 2·9 nM resulted in protection of the *ara*I region. The appearance of the footprint at *ara*I corresponded approximately to the stimulation of transcription in the absence of CRP as shown in Figure 6. Lee *et al.* (1981) examined protection of the *ara*I site at concentrations of AraC protein only above 10 nM. Under such conditions, they also found a footprint of the *ara*I site, and at 10 nM, transcription from P_{BAD}. No parallel transcription–binding experiments of P_{BAD} were reported.

In our experiments when CRP was present, no significant change in the level of AraC protein necessary for DNase I protection was seen. Thus, the presence of CRP does not appear to alter the mode of AraC binding that is known to be occurring both by the gel binding assay and by the transcription results.

In conclusion, the results of the experiments performed with linear DNA template indicate that AraC protein can stimulate transcription from P_{BAD} on a linear template in two ways. At low concentrations of AraC protein in the presence of arabinose, the protein binds to the *ara*I site, but requires CRP in order to be functional. At high concentrations, the AraC also binds, but it appears to contact DNA differently, and does not require CRP in order to activate transcription from P_{BAD}.

4. Discussion

We have examined the role of CRP in the induction of the *ara*BAD promoter P_{BAD}. Using supercoiled DNA templates, we found conditions that reproduced the behavior of the promoter *in vivo* with respect to induction by arabinose, the requirement for CRP for induction, and the effect of the upstream AraC protein binding site named *ara*O_2.

In addition to its inhibition of *ara*O_2 function, we found that CRP can play a second role in stimulating formation of open complexes of RNA polymerase on P_{BAD}. This occurs at lower concentrations of AraC protein, does not involve *ara*O_2, and does not require supercoiled DNA. Finally, experiments with linear DNA are presented that show that, under the experimental conditions, the binding of a single molecule of AraC protein to *ara*I in the absence of CRP is insufficient to stimulate open complex formation at P_{BAD}. Either CRP or a second molecule of AraC protein must be involved. The presence of this additional molecule of AraC protein appears to alter the contacts made by the first molecule to bind to the DNA.

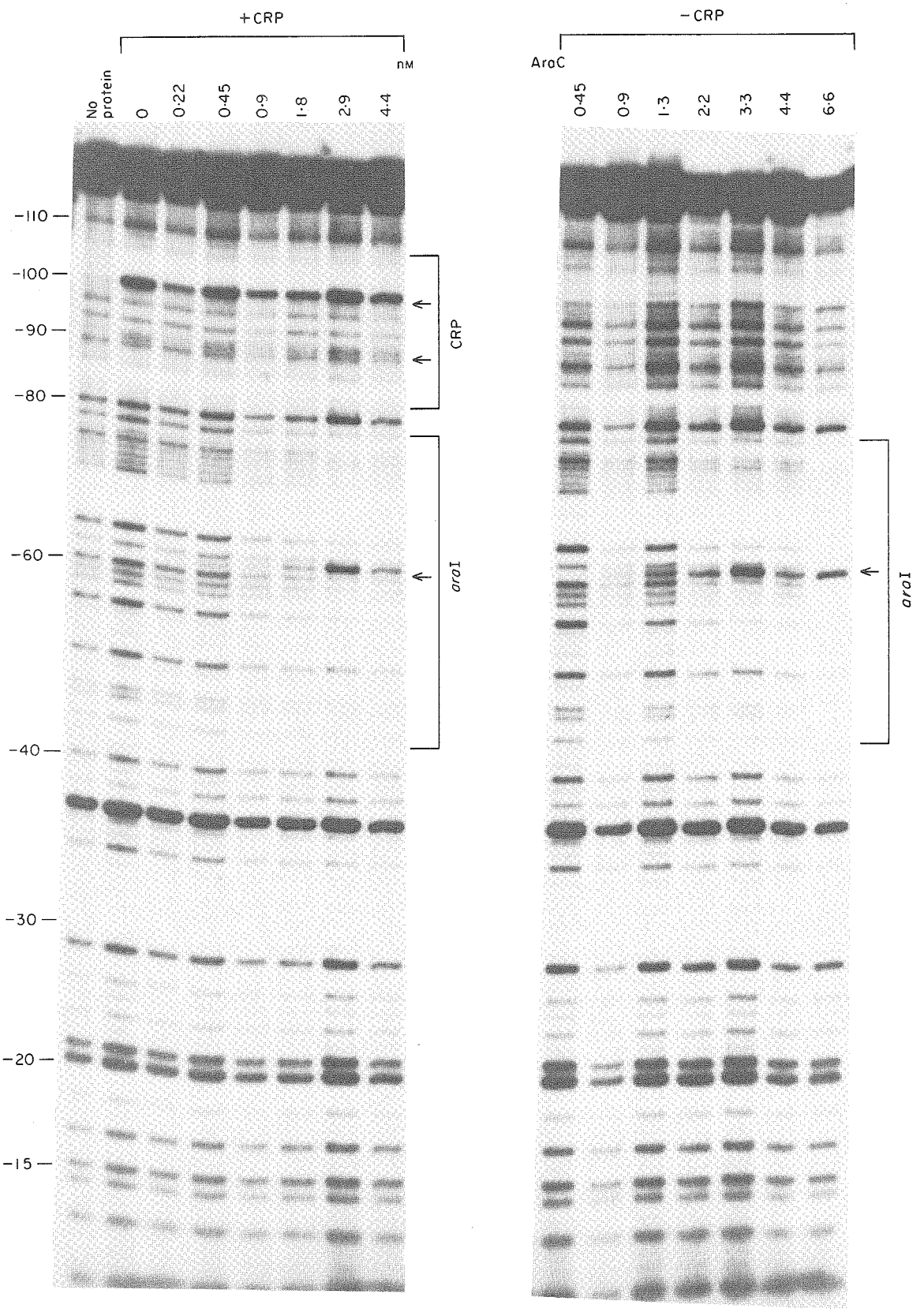

Figure 8. High levels of AraC protein are required for DNase I protection at *ara*I. DNase I footprinting was carried out under conditions identical to those for Figs 6 and 7. Reactions contained 0·15 nM-linear pΔ383 DNA and 0·15 nM-(γ-^{32}P)-labeled Δ383 DNA fragment in standard transcription buffer, pH 7·8, 37°C. CRP (5 nM), where indicated, and various amounts of AraC protein were incubated with the DNA for 10 min followed by a 15 s DNase I digestion. Brackets mark the position of the *ara*I site and the CRP site defined previously by DNase I protection (Ogden *et al.*, 1980; Lee *et al.*, 1981). The arrows mark positions of enhanced DNase I cleavage. The numbers mark the position (in base-pairs) relative to the start of P_{BAD} transcription.

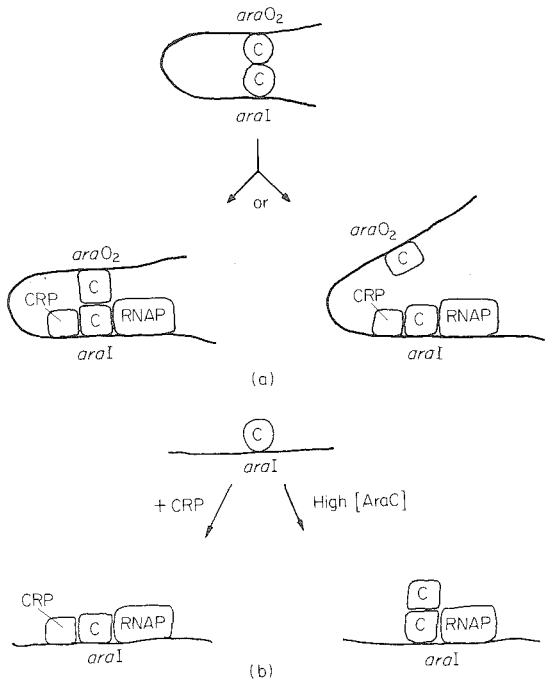

Figure 9. Models for regulation of P_{BAD}. (a) Model for repression and CRP activation of P_{BAD}. In the absence of CRP, AraC–arabinose bound at both $araO_2$ and *araI* interacts *via* a loop in the intervening DNA. Under these conditions, AraC exists in a state unable to stimulate P_{BAD} transcription (○). Upon addition of CRP, AraC is converted to the active state (□), which may abolish the interaction between $araO_2$ and *araI*. (b) Model for induction of P_{BAD} on a template deleted for $araO_2$. A single molecule of AraC–arabinose at *araI* exists in a state unable to stimulate P_{BAD} transcription (○). Addition of CRP converts AraC into the active state (□). Similarly, at high levels of AraC protein, a second molecule of AraC–arabinose interacts at some site downstream from DNA position −84, here hypothetically drawn as AraC itself, to stimulate transcription.

In a series of experiments *in vivo*, repression in the *ara* system has been shown to require the $araO_2$ site (Dunn *et al.*, 1984; Hahn *et al.*, 1984). We wished to study repression and the role of $araO_2$ *in vitro*. However, repression in the *ara* system is the ability of AraC protein in the absence of arabinose to keep the basal level of P_{BAD} expression low and, in the absence of arabinose, to play an active role in reducing the expression of a mutant constitutive P_{BAD} promoter. Examination of either of these phenomena *in vitro* is difficult, due to the fact that both involve low levels of transcription.

A way to study the mechanism of $araO_2$ in transcription appeared with the discovery that when $araO_2$ is deleted, the requirement for CRP for induction of P_{BAD} is greatly alleviated (Hahn *et al.*, 1984). We found this property could be reproduced by the use of supercoiled DNA template in our transcription system *in vitro*. Furthermore, the behavior of P_{BAD} *in vivo* as a result of altering the spacing and hence angular orientation between $araO_2$ and *araI* *in vivo* suggests that these two regions interact *via* a loop in the DNA. We found a similar response *in vitro*, also suggesting that the regions interact *via* a DNA loop. Overall, the similarity of the results *in vivo* and *in vitro* strengthens the conclusion that the phenomenon observed involves a DNA loop, because the defined system eliminates the possibility of additional interactions with other unknown components *in vivo* that could generate the observed phenomena by alternative mechanisms.

Since the two classical manifestations of repression in the *ara*BAD system involve $araO_2$ in the same way as the recently discovered phenomenon of CRP-independent P_{BAD} induction, it seems reasonable to suggest that all three phenomena possess the same physical basis.

One mechanism consistent with the known information is that, in the absence of arabinose, AraC protein is bound at $araO_2$ and at *araI*, and that the DNA between these sites loops so that the proteins bound to both are in direct contact (Fig. 9(a)). This configuration could help hold AraC protein in a repressing conformation, from which it plays an active role in blocking transcription from the adjacent RNA polymerase binding site of P_{BAD}. If arabinose is added but CRP or cyclic AMP is absent, little induction occurs. However, if CRP and cyclic AMP are present also, the repression engendered by the loop is overcome and AraC protein assumes an inducing conformation. It then stimulates initiation of transcription from P_{BAD}. It is premature to speculate whether the hypothesized loop actually opens in this induction process.

Repression *in vivo* can be prevented by mutations in $araO_2$ that prevent binding by AraC protein (Kathy Martin, personal communication) and a mutation in *araI* (Hahn *et al.*, 1984), by deletion of $araO_2$, or by spacing mutations between $araO_2$ and *araI* such that loop formation would require twisting the DNA by half a turn (Dunn *et al.*, 1984). In the absence of the postulated loops, CRP no longer would be required for induction, just as is observed *in vivo*, and *in vitro* at high concentrations of AraC.

The experiments discussed above show little CRP stimulation from linear DNA templates, and yet reports have been published that P_{BAD} contained on a linear template is stimulated by CRP (Hirsh & Schleif, 1977; Lee *et al.*, 1974). In addition to investigating the source of the discrepancy between earlier work and the present experiments, we wished to perform experiments in which we could simultaneously measure protein binding as assayed by the gel electrophoresis binding assay and transcription, and determine the region of DNA to which the protein binds by DNase footprinting.

At lower concentrations of AraC protein than those used in our experiments discussed above, we did find that CRP can stimulate transcription from P_{BAD} when it is located on a linear template. Under the conditions of the experiments described here, AraC protein binds at 0·45 nM to virtually all the P_{BAD} DNA molecules. Despite its binding as shown by the gel electrophoresis assay, this protein does

not activate transcription unless CRP is present. Only when the concentration of AraC protein is increased does AraC protein–arabinose alone become capable of stimulating open complex formation on P_{BAD} (Fig. 9(b)).

There is no reason to think that the gel assay of binding and the transcription experiments are giving misleading answers about binding and activity. Consequently, we infer that the binding of a single molecule of AraC protein to *ara*I under the conditions of the experiments does not stimulate formation of open complexes at P_{BAD}. Only on the binding of a second molecule of AraC can the protein stimulate RNA polymerase.

The DNase protection experiments were consistent with the picture generated by the gel binding measurements and transcription measurements. In a parallel binding and DNase footprinting experiment, the binding of the first molecule of AraC protein did not protect the *ara*I site from digestion by DNase, and only one position in the site shows an increased cleavage frequency in the presence of AraC protein. The presence of CRP in the binding does not alter the pattern of DNase digestion, but it does permit the AraC protein at these concentrations to activate transcription. However, increasing the concentration of AraC protein alters the protection pattern so that a footprint becomes visible. This occurred at a concentration at which transcription began to appear.

Miyada *et al.* (1984) and Horwitz *et al.* (1984) have proposed that CRP stimulates P_{BAD} less than fivefold *in vivo* and that this stimulation derives from a site other than the CRP binding site located about 80 nucleotides upstream from P_{BAD}. This conclusion was based on the observations that (1) a three base-pair deletion within the CRP binding site did not significantly reduce P_{BAD} activity *in vivo*, and (2) that P_{BAD} expression was repressed four- to fivefold by glucose in both the wild-type and the strain deleted in the CRP binding site. Catabolite repression by glucose should not be used as a criterion for a functional CRP site as was used by Miyada *et al.* (1984) since both *lac* and P_{BAD} are stimulated 20 to 40-fold by CRP-cAMP *in vitro* and *in vivo* (Beckwith *et al.*, 1972; Lis & Schleif, 1973; Heffernan *et al.*, 1976) but repression by glucose is only three- to fourfold (Wanner *et al.*, 1978; Heffernan *et al.*, 1976). This three- to fourfold repression by glucose cannot be overcome completely by the addition of cAMP (Wanner *et al.*, 1978; Heffernan *et al.*, 1976). Finally, while the wild-type P_{BAD} is 2 to 5% of fully inducible in the absence of CRP, deletion of *ara*O_2 leaves the promoter greater than 50% of fully inducible in the absence of CRP (Hahn *et al.*, 1984; Wilcox *et al.*, 1982), but such a deletion has no effect on the fourfold repression by glucose (Bass *et al.*, 1976).

The fact that a three base-pair deletion at the CRP site has only a small effect on P_{BAD} can be explained by the experiments *in vitro* reported here. Insertion or deletion of less than an integral number of helical turns between *ara*O_2 and *ara*I can greatly reduce the CRP requirement for P_{BAD}. Based on these results, a three base-pair deletion in the CRP binding site might have little effect on induced P_{BAD} expression, since it should also reduce the CRP requirement *in vivo*.

In summary, regulation of P_{BAD} transcription is under both positive and negative control, and the mechanisms responsible apparently interact in a complex manner. DNA looping is a mechanism that can economically explain much of the known data in which the function of CRP could be to help overcome the repression generated by the loop between *ara*O_2 and *ara*I. It would not be necessary for CRP to be located adjacent to the RNA polymerase in order to activate transcription by this mechanism. Such a proposal is related to the finding (Malan & McClure, 1984) that one function of CRP in the *lac* system is to block non-productive binding by RNA polymerase to a second promoter. However the *ara* system shows additional complexity in the fact that under some conditions *in vitro*, two mechanisms exist for stimulation by AraC protein. One mechanism requires both AraC and CRP, the other requires only AraC protein.

We thank Charles Yanofsky for his generous gift of the *rpo*C terminator. We thank Pieter Wensink and members of this laboratory for comments on the manuscript. This work was supported by U.S. Public Health Service Research grant GM18277 from the National Institutes of Health.

References

Bass, R., Heffernan, L. & Englesberg, E. (1976). *Arch. Microbiol.* **110**, 135–143.

Beckwith, J., Grodzicker, T. & Arditti, R. (1972). *J. Mol. Biol.* **69**, 155–160.

Brown, P. (1979). Ph.D. thesis, Brandeis University.

Burgess, R. & Jendrisak, J. (1975). *Biochemistry*, **14**, 4634–4638.

Cossart, P. & Gicquel-Sanzy, B. (1982). *Nucl. Acids Res.* **10**, 1364–1378.

de Crombrugghe, B., Busby, S. & Buc, H. (1984). *Science*, **221**, 831–838.

Dunn, T. & Schleif, R. (1984). *J. Mol. Biol.* **180**, 201–204.

Dunn, T., Hahn, S., Ogden, S. & Schleif, R. (1984). *Proc. Nat. Acad. Sci., U.S.A.* **81**, 5017–5020.

Eilen, E., Panpeno, C. & Krakow, J. (1978). *Biochemistry*, **17**, 2469–2473.

Englesberg, E., Irr, J., Power, J. & Lee, N. (1965). *J. Bacteriol.* **99**, 946–947.

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

Englesberg, E., Sheppard, D., Squires, C. & Meronk, F. J. (1969*b*). *J. Mol. Biol.* **43**, 281–298.

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

Hahn, S. & Schleif, R. (1983). *J. Bacteriol.* **155**, 593–560.

Hahn, S., Dunn, T. & Schleif, R. (1984). *J. Mol. Biol.* **180**, 61–72.

Heffernan, L., Bass, R. & Englesberg, E. (1976). *J. Bacteriol.* **126**, 1119–1131.

Hendrickson, W. & Schleif, R. (1984). *J. Mol. Biol.* **174**, 611–628.

Hendrickson, W. & Schleif, R. (1985). *Proc. Nat. Acad. Sci., U.S.A.* **82**, 3129–3133.
Hirsh, J. & Schleif, R. (1977). *Cell*, **11**, 545–550.
Horwitz, A., Miyada, C. & Wilcox, G. (1984). *J. Bacteriol.* **158**, 141–147.
Irani, M. H., Orosz, L. & Adhya, S. (1983). *Cell*, **32**, 783–788.
Kolodrubetz, D. & Schleif, R. (1981). *J. Mol. Biol.* **151**, 215–227.
Kosiba, B. & Schleif, R. (1982). *J. Mol. Biol.* **156**, 53–66.
Lee, N., Wilcox, G., Gielow, W., Arnold, J., Cleary, P. & Englesberg, E. (1974). *Proc. Nat. Acad. Sci., U.S.A.* **71**, 634–638.
Lee, N., Gielow, W. & Wallace, R. (1981). *Proc. Nat. Acad. Sci., U.S.A.* **78**, 752–756.
Levine, A. & Rupp, W. (1978). In *Microbiology* (1978) (Schlessinger, D., ed.), pp. 163–166, American Society for Microbiology, Washington, D.C.
Lis, J. & Schleif, R. (1973). *J. Mol. Biol.* **79**, 149–162.
Lowe, P., Hager, D. & Burgess, R. (1979). *Biochemistry*, **18**, 1344–1352.
Maizels, N. (1973). *Proc. Nat. Acad. Sci., U.S.A.* **70**, 3585–3589.
Malan, T. & McClure, W. (1984). *Cell*, **39**, 173–180.
Maniatis, T., Fritsch, E. & Sambrook, J. (1982). *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
Miyada, G., Stoltzfus, L. & Wilcox, G. (1984). *Proc. Nat. Acad. Sci., U.S.A.* **81**, 4120–4124.
Morita, M. & Oka, A. (1979). *Eur. J. Biochem.* **97**, 435–443.
Ogden, S., Haggerty, D., Stoner, C., Kolodrubetz, D. & Schleif, R. (1980). *Proc. Nat. Acad. Sci., U.S.A.* **77**, 3346–3350.
Reiner, A. (1969). *J. Bacteriol.* **97**, 1437–1443.
Rosenberg, M., Chepelinsky, A. & McKenney, K. (1983). *Science*, **222**, 734–739.
Schleif, R. & Favreau, A. (1982). *Biochemistry*, **21**, 778–782.
Schleif, R. & Wensink, P. (1981). *Practical Methods in Molecular Biology*, Springer-Verlag, New York.
Squires, C., Kramer, A., Barry, G., Shen, W. & Squires, G. (1981). *Nucl. Acids Res.* **9**, 6827–6840.
Steffen, D. & Schleif, R. (1977). *Mol. Gen. Genet.* **157**, 333–339.
Stoner, C. & Schleif, R. (1983). *J. Mol. Biol.* **170**, 1049–1053.
Wanner, B., Kodaira, R. & Neidhart, F. (1978). *J. Bacteriol.* **136**, 947–954.
Wilcox, G., Meuris, P., Bass, R. & Englesberg, E. (1974). *J. Biol. Chem.* **249**, 2946–2952.
Wilcox, G., Al-Zarban, S., Cass, L. G., Clarke, P., Heffernan, L., Horwitz, A. H. & Miyada, C. G. (1982). In *Promoters: Structure and Function* (Rodriguez, R. & Chamberlin, M., eds), pp. 183–194, Praeger Scientific Publishing Co., New York.
Yu, N. & Reznikoff, W. (1984). *Nucl. Acids Res.* **12**, 5449–5466.

Edited by I. Herskowitz