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Regulation of the acetate operon in *Escherichia coli*: purification and functional characterization of the IclR repressor

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Growth of *Escherichia coli* on acetate requires operation of the anaplerotic sequence known as the glyoxylate bypass. In this pathway three different enzymes are activated: malate synthase, isocitrate lyase and isocitrate dehydrogenase kinase/phosphatase which are encoded by genes *aceB*, *aceA* and *aceK*, respectively. These three genes are clustered, in that order, in the same acetate (*ace*) operon whose expression is under the transcriptional control of the *iclR* gene located downstream from *aceK*. We have cloned the *iclR* gene in the pKK233-2 vector which allows optimization of both transcription and translation initiation. The IclR repressor has been overproduced, then purified to homogeneity in a one-step procedure by cation exchange chromatography after ammonium sulfate fractionation. Its specific interaction with the operator/promoter region of the *ace* operon has been analyzed by gel retardation and DNase I footprinting experiments. The IclR repressor has been shown to recognize a 35 bp palindromic sequence which largely overlaps the −35 recognition site of RNA polymerase. Moreover, the formation of the complex between IclR and the operator/promoter region has been found to be impaired by phosphoenol pyruvate but insensitive to acetate, acetyl-CoA, pyruvate, and oxaloacetate. These results are discussed in terms of primary regulation of the expression of the *ace* operon.

Key words: acetate operon/DNA−repressor complex/promoter−operator/protein phosphorylation

Introduction

When acetate becomes the sole source of carbon, *Escherichia coli* cells adjust by inducing two new enzymes, isocitrate lyase and malate synthase, which divert some of the carbon flux from the Krebs cycle into the glyoxylate bypass (Kornberg, 1966; Lakshmi and Helling, 1978). Bacteria are thus provided with the 4-carbon and 3-carbon intermediates required for the biosynthetic pathways (Holms and Bennett, 1971; Andersen and Von Meyenburg, 1980; Nimmo *et al.*, 1988). The channeling of metabolites from the Krebs cycle into the glyoxylate cycle occurs at the level of isocitrate. It is connected with the regulation of isocitrate dehydrogenase (IDH) activity by reversible phosphorylation. Indeed, when *E.coli* cells are grown on acetate (or fatty acids) IDH becomes phosphorylated and concomitantly its activity declines drastically (Garnak and Reeves, 1978; Borthwick *et al.*, 1984). Conversely, when bacteria are cultured on carbon sources such as glucose or glycerol which do not employ the glyoxylate bypass as an anaplerotic sequence, IDH is not phosphorylated and remains fully active. The phosphorylation−dephosphorylation of IDH is mediated by a bifunctional enzyme, IDH kinase/phosphatase, which contains both modifying and demodifying activities on the same polypeptide encoded by a single gene (LaPorte and Koshland, 1982; LaPorte and Chung, 1985).

The mechanisms that regulate the expression of the glyoxylate bypass remain unclear. Genetic studies indicate that the structural genes coding for malate synthase (*aceB*), isocitrate lyase (*aceA*), and IDH kinase/phosphatase (*aceK*) are present, in that order, in the same acetate (*ace*) operon located at 90 min on the *E.coli* K-12 linkage map (reviewed by Cortay *et al.*, 1989). The expression of this operon is negatively controlled at transcription by two gene products: that of the *iclR* gene, which is located downstream from the *aceK* gene, and that of the *fadR* gene, which maps at 25 min and also participates in the regulation of the fatty acid metabolism involving the *fad* and *fab* regulons (Maloy and Nunn, 1982; Nunn, 1986). Merodiploid studies have shown that both the *iclR* and *fadR* gene products regulate the *ace* operon in a *trans*-dominant manner (Maloy and Nunn, 1982). Recently, the nucleotide sequence of the *iclR* genes from both *E.coli* and *Salmonella typhimurium* has been determined (Galinier *et al.*, 1990; Nègre *et al.*, 1991; Sunnarborg *et al.*, 1990).

In this work the *iclR* gene of *E.coli* K-12 has been cloned and overexpressed in the pKK233-2 vector which allows optimization of both transcription and translation initiation (Amann and Brosius, 1985). In these conditions it has been possible to produce the IclR repressor in quantities sufficient to purify it to homogeneity, and to study its specific interaction with the operator/promoter region of the *ace* operon.

Results

Cloning and overexpression of the iclR gene

In order to produce relatively large amounts of IclR repressor, the *E.coli iclR* gene was cloned into plasmid pKK233-2 (Amann and Brosius, 1985). This expression vector (Figure 1) contains the strong *trc* promoter which is a hybrid form of *trp* and *lac* promoters, with the consensus 17 bp spacing between the *trp*-35 region and the *lac* UV5-10 region. It also possesses the *lacZ* ribosome binding site (RBS) and an ATG initiation codon located at the optimal distance of 8 bp from the RBS within a unique *Nco*I restriction site (CCATGG). Located just downstream from this *Nco*I site is a unique *Pst*I recognition sequence. The *iclR* gene was inserted into pKK233-2, after PCR amplification (see Materials and methods), by using two synthetic DNA linkers which created an *Nco*I site overhang at the 5'-end of *iclR*

and a *Pst*I site at the 3′-end. The resulting plasmid pJCAD6 (Figure 1) was used to overproduce the IclR protein in the *lacI*^{q} host strain JM105 after induction by IPTG. The presence of IclR among the synthesized proteins was detected by SDS−PAGE (Figure 1). By comparing its mobility with that of reference proteins of known molecular mass, the size of IclR in its monomeric form was found to be ~30 kd, which is in agreement with the value deduced from the nucleotide sequence of the *iclR* gene (Nègre *et al.*, 1991; Sunnarborg *et al.*, 1990).

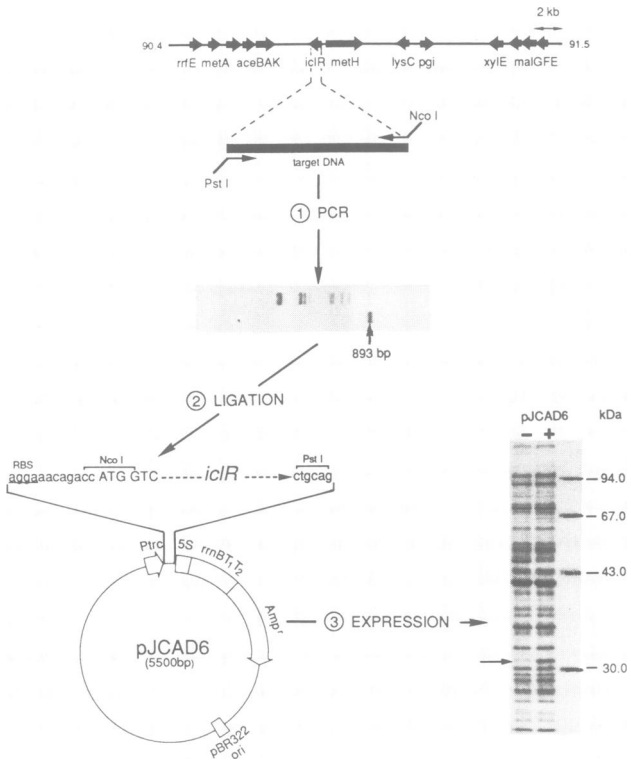

Fig. 1. Construction of plasmid pJCAD6 and expression of the *iclR* gene. The *iclR* gene was amplified from the *E.coli* chromosome by PCR using two oligonucleotide primers containing respectively a *Nco*I and a *Pst*I restriction site at their 5′ end. An 893 bp fragment was obtained, separated by agarose gel electrophoresis and ligated into vector pKK233-2 to yield plasmid pJCAD6. The expression of pJCAD6 was studied in the host strain JM105. The *iclR* gene product was detected in the 30 kd region (arrow) after SDS−PAGE.

Purification of the IclR repressor

To investigate precisely the interaction of the repressor IclR with its specific operator region, it was essential to obtain this protein in a purified form. For this purpose, IclR was overproduced from plasmid pJCAD6 as indicated above, host cells JM105 were collected, ground in alumina, and a total protein fraction (S225) was prepared by high-speed centrifugation. Purification of IclR was obtained by ammonium sulfate fractionation of this fraction followed by cation exchange chromatography using an FPLC system (see Materials and methods). The corresponding elution profile (Figure 2) shows that IclR was obtained as a single peak (shown by arrow) eluted by 0.7 M ammonium chloride. Analysis of the protein material of this peak by SDS−PAGE revealed only one band, with a molecular mass of ~30 kd.

Functional characterization of the IclR repressor

When DNA is interacting with protein, the corresponding complex exhibits a significantly slower electrophoretic mobility in non-denaturing polyacrylamide gels than free DNA. This property has been successfully utilized to demonstrate the specific binding of proteins to DNA (Fried and Crothers, 1981; Garner and Revzin, 1981). We performed such gel retardation experiments to evaluate the specific binding of IclR to the operator/promoter region of the *ace* operon. A *Hinc*II−*Eco*RI DNA fragment of 197 bp containing this region (Byrne *et al.*, 1988; Chung *et al.*, 1988) was prepared, labeled at the *Eco*RI site by radioactive dATP, and purified by gel filtration. It was then incubated with an increasing amount of pure IclR repressor and electrophoresed in non-denaturing conditions. Figure 3 shows that, after autoradiography, one could detect a progressive reduction of the amount of free DNA and a concomitant increase of the slowly migrating band corresponding to DNA−protein complex (lanes 2−4).

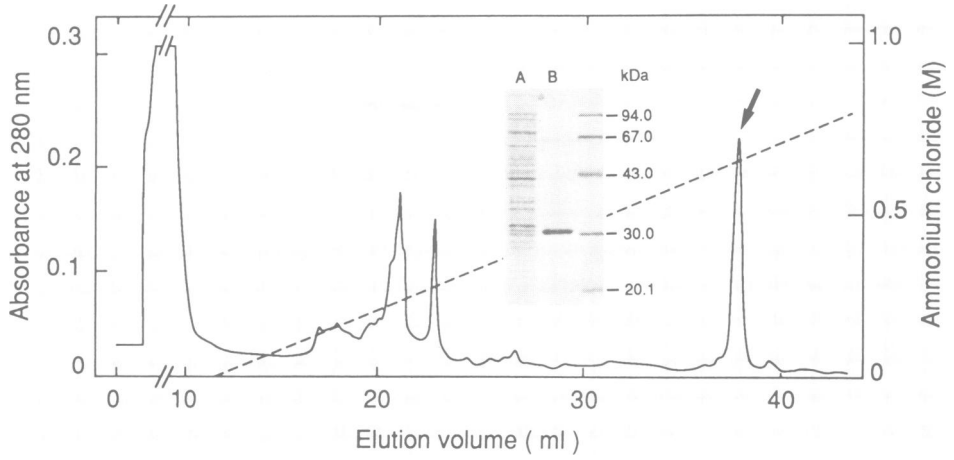

Fig. 2. Purification of the IclR repressor by chromatography. A high-speed S225 fraction was prepared from JM105 cells transformed by plasmid pJCAD6. It was fractionated by ammonium sulfate as indicated in Materials and methods, and loaded on an FPLC cation exchange column SP-5PW. Elution was achieved with a 0−1 M ammonium chloride gradient (dotted line). The total protein preparation loaded on the column (lane A) and the pooled fractions corresponding to IclR (lane B) were analyzed by polyacrylamide gel electrophoresis. Reference proteins of known molecular mass were run in parallel.

However, at a higher concentration of IclR (lane 5) no more DNA could be complexed, suggesting that the binding site was saturated. These observations indicate that the IclR repressor is capable of interacting in a specific manner with the operator/promoter region of the *ace* operon.

The binding of IclR to DNA was investigated further by carrying out DNase I footprinting experiments. The labeled 197 bp DNA fragment containing the operator/promoter region was incubated with IclR repressor and partially digested with DNase I. The products were fractionated according to size by gel electrophoresis and visualized by autoradiography. In the absence of repressor, DNase I cleavage produced a distinct pattern of bands (Figure 4, upper lane). Upon addition of the IclR repressor (lower lane) a protected region of 35 bp appeared, covering nearly all the palindromic structure present in this particular region of DNA (shown by horizontal arrows). Of particular interest was the finding that the region protected by IclR largely overlaps the −35 region for RNA polymerase attachment (Figure 4). In other words the operator and the promoter sequences are, in this case, essentially coincident.

Effect of metabolites on the DNA − IclR complex

An attempt was made to identify some of the metabolite(s) that might be involved in the control of the *ace* operon expression. For this, the effect of five different molecules on the formation of the IclR−DNA complex was analyzed.

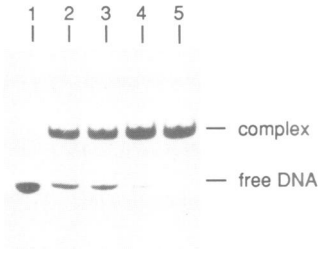

Fig. 3. Binding of the IclR repressor to the operator of the *ace* operon. A 197 bp DNA fragment containing the operator region of the *ace* operon was prepared as described in Materials and methods. It was radioactively labeled with [α-^{32}P]dATP and incubated with an increasing concentration of pure IclR repressor varying from 1 to 120 nM. After 10 min of incubation, the mixture was loaded onto a 4% polyacrylamide gel at low ionic strength and electrophoresed. Radioactive compounds were detected by autoradiography, either as free or complexed DNA. Lane 1: DNA alone; lanes 2−5: DNA with 1, 10, 50 or 120 nM IclR, respectively.

These include acetate, acetyl-CoA, pyruvate, phosphoenol pyruvate and oxaloacetate, whose capacity to affect the binding of IclR to the operator/promoter region was tested by gel retardation assays. Figure 5 shows that among these various molecules only phosphoenol pyruvate (lane 2) appeared to prevent the formation of the IclR−DNA complex and thus seems able to play a positive role in the expression of the *ace* operon. Identical results were obtained when using each metabolite at a final concentration of either 4 μM (Figure 5) or 40 μM (not shown). In addition, no effect on the formation of the IclR−DNA complex was observed in the presence of acetyl phosphate, a metabolic intermediate between acetate and acetyl-CoA.

Discussion

A number of metabolic processes in prokaryotes have recently been shown to be regulated by reversible protein phosphorylation (reviewed by Cozzone, 1988, and Stock *et al.*, 1989). One of them concerns the partition of carbon molecules between the Krebs cycle and the glyoxylate cycle in acetate-grown bacteria. The general mechanism that modulates the activity of the key enzyme, isocitrate dehydrogenase, at the branch point between the two cycles is well established (Garnak and Reeves, 1978; Nimmo *et al.*, 1988), but no satisfactory explanation has been given as yet for the molecular processes that control the expression of the *ace* operon. Obviously an important step in the elucidation of this problem will be the characterization of the structure and mode of action of the effectors, negative and positive, involved in the functioning of the operon.

Within this scope, we have focused our attention on the IclR repressor which negatively controls the *ace* operon at the level of transcription (Maloy and Nunn, 1982). In the present work we have overproduced the IclR protein by making use of plasmid pKK233-2, which allows optimization of transcription as well as translation initiation (Amann and Brosius, 1985). Then, for the first time, IclR has been purified to homogeneity, as judged by electrophoretic analysis, by using a one-step chromatographic procedure. It would be interesting to check whether other repressor proteins, namely FadR, could also be purified by this efficient procedure.

The analysis of the interaction between IclR and the operator/promoter region of the *ace* operon has shown that the repressor binds specifically to a 35 bp sequence which overlaps almost completely the −35 binding site of RNA polymerase. Such a topological coincidence between the

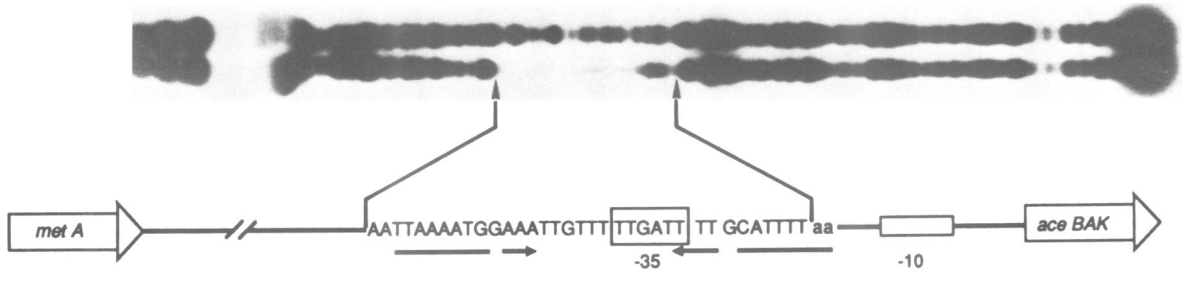

Fig. 4. Nucleotide sequence of the *ace* operator. A 197 bp DNA fragment containing the operator region of the *ace* operon, between the *metA* and *aceB* genes, was radioactively labeled at the 3′ end and subjected to partial DNase I digestion in the presence of pure IclR repressor. The region protected by IclR against hydrolysis is indicated between two vertical arrows. The location of the −10 and −35 binding regions of RNA polymerase is presented in boxes. The horizontal arrows under the nucleotide sequence show a palindromic structure.

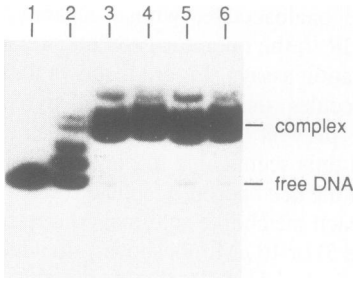

Fig. 5. Effect of various metabolites on the IclR–operator interaction. A 197 bp radioactive fragment of DNA containing the operator of the *ace* operon was incubated with the IclR repressor (120 nM) in the presence, or not, of various metabolites (4 μM each) individually. In each case the formation of a DNA–IclR complex was detected by autoradiogaphy after gel electrophoresis. Lane 1: control DNA alone; lane 2: incubation with phosphoenol pyruvate; lanes 3–6: incubation with acetate, acetyl-CoA, pyruvate and oxaloacetate, respectively.

operator and promoter regions is rather unusual. To our knowledge, a similar situation has been so far reported only in the case of the *aroG/aroH* loci involved in the biosynthesis of aromatic amino acids (Baseggio *et al.*, 1990). This observation indicates that the repressor and the transcription enzyme will compete for the same binding site on DNA, and reinforces the concept that repression is a matter of blocking access to the promoter. It is noticeable, however, that we did not detect any protection in the −10 region.

The intracellular signal for induction of the glyoxylate bypass remains to be elucidated. Several observations suggest, but do not demonstrate, that the expression of the *ace* operon does not simply respond to the presence of acetate or acetyl-CoA. Thus, when bacteria are grown simultaneously on acetate and on a preferred carbon source such as glucose, operation of the glyoxylate bypass does not occur, even though the capacity of cells to convert acetate into acetyl-CoA in these conditions remains nearly the same as during growth on acetate only. In addition, when bacteria are cultured on fatty acids, a carbon source whose utilization does not proceed through acetate, the glyoxylate bypass is nevertheless induced (Nunn, 1986). Our results bring the first direct evidence that the *ace* operon does not respond to acetate itself. Neither does it respond to acetyl-CoA, pyruvate or oxaloacetate. But the formation of the DNA–IclR complex is, by contrast, impaired by phosphoenol pyruvate, which therefore seems to be a good candidate as an inducer of the operon transcription. Further studies are however required to check this possibility.

The product of the *fadR* gene is known to act as a repressor not only for the *fad* regulon, but also for the *ace* operon (Nunn *et al.*, 1979; Simons *et al.*, 1980). Both *iclR* and *fadR* mutants express elevated levels of *ace* enzymes during growth on succinate, a carbon source that significantly represses expression in wild-type cells (Maloy and Nunn, 1982). Moreover, expression of the *ace* operon in these culture conditions is even greater in *iclR fadR* double mutants. One possible interpretation of these results could be that the *iclR* and *fadR* gene products act independently to cause the partial repression of the operon; when acting together, they cause its full repression (Maloy and Nunn, 1982). It is not known, however, whether the *fadR* gene product would exert control over the *ace* operon by directly interacting with *cis*-acting regulator sites on the operon or by interacting with the *iclR* gene product (Nunn, 1987). By extending to the FadR repressor the type of analysis presented here for IclR and by investigating the respective role and mode of action of each repressor, one can expect to get more insight soon into the molecular mechanism that regulates the expression of the *ace* operon.

Materials and methods

Amplification of iclR gene from E.coli chromosomal DNA

The *iclR* gene was specifically amplified by the polymerase chain reaction (PCR) technique using two primers containing respectively a *Nco*I and *Pst*I site at their 5′ end. Chromosomal DNA was prepared by the method of Ardeshir *et al.* (1981).

The PCR reaction (Saiki *et al.*, 1988) was carried out in a 100 μl final volume containing 1 μg of chromosomal DNA, 10 mM Tris–HCl (pH 8.3 at room temperature), 1.5 mM magnesium chloride, 50 mM potassium chloride, 0.01% w/v gelatin, 200 μM of each deoxynucleoside triphosphate, 1 μM oligomers, and 2.5 U of *Thermus aquaticus* DNA polymerase. The amplification reaction was carried out for 30 cycles in a DNA thermal cycler (Perkin Elmer-Cetus). The DNA was denatured at 94°C for 1 min, annealed at 55°C for 1 min, and extended at 72°C for 2 min. To analyze the amplification products, 2 μl of the reaction mixture was electrophoresed on an 0.8% agarose gel and visualized with ethidium bromide.

Construction and expression of plasmid pJCAD6

The 893 bp fragment obtained by PCR amplification was digested with *Nco*I and *Pst*I nucleases, and ligated into pKK233-2 (Amann and Brosius, 1985) previously opened with the same enzymes. The resulting plasmid pJCAD6 carried the *iclR* gene under the control of the *trc* promoter. Its expression was analyzed after transformation of the host strain JM105 *thi, rps* L, *end*A, *sbc*B15, *hsd*R4, Δ(*lacpro*AB)/F′, *tra*D36, *pro*AB, *lacI*^{q}ΔM15.

Preparation of the operator/promoter DNA fragment

A 165 bp fragment containing the promoter/operator region of the *ace* operon (−188 to −23 from the ATG of the *aceB* gene) (Byrne *et al.*, 1988; Chung *et al.*, 1988) was prepared by digesting pMA6 (Michaeli and Ron, 1984) with *Rsa*I and *Bam*HI. The corresponding fragment was cloned into plasmid pUC18, digested by *Sma*I + *Bam*HI, to yield pJCAD8. A *Hinc*II–*Eco*RI fragment of 197 bp was then excised from pJCAD8 and labeled at the *Eco*RI site by [α-^{32}P]dATP (3000 Ci/mmol, Amersham Corp.) with the Klenow fragment of DNA polymerase I. This labeled fragment containing the *ace* promoter region was further purified by Sephadex-G50 chromatography and incubated with purified IclR repressor for gel retardation analysis.

Purification of the iclR repressor

Strain JM105 transformed by plasmid pJCAD6 was grown exponentially at 37°C in rich TB medium containing 25 μg/ml streptomycin and 50 μg/ml ampicillin (Tartof and Hobbs, 1987). At the optical density of 0.5–0.6 at 600 nm, cells were induced by adding IPTG to a final concentration of 1 mM and incubation was continued for 4 h. Bacteria were harvested by low-speed centrifugation, disrupted by alumina grinding, and suspended in a buffer containing 20 mM HEPES–KOH pH 7.5, 60 mM KCl, 10 mM $MgCl_2$, 4 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF). A high-speed supernatant (S225) was then prepared by centrifugation at 225 000 *g*.

Solid ammonium sulfate was added to S225 to 40% saturation at 0°C. The suspension was centrifuged and the pellet was dissolved in buffer A (0): 20 mM HEPES–KOH pH 7.5, 1 mM EDTA, 2 mM DTT and 0 mM NH_4Cl and dialyzed overnight against the same buffer with three changes. Final purification was achieved on a cation-exchange column (Waters-Protein Pak Glass SP-5PW, 7.5 × 5 cm). Elution was performed with A(0)–A(1000)NH_4Cl gradient. Each peak was analyzed by SDS–polyacrylamide (12.5%) gel electrophoresis. The fractions containing the IclR protein were pooled and concentrated with a Centricon-10 filtration device (Amicon). Protein concentration was determined spectrophotometrically according to Bradford (1976).

Gel retardation analysis

Gel retardation assays were performed essentially as described by Garner and Revzin (1981). A typical assay mixture contained in 50 μl: 12 mM HEPES–NaOH pH 7.9, 4 mM Tris–HCl pH 7.9, 95 mM KCl, 1 mM EDTA, 1 mM DTT, 12% glycerol, 0.5 μg poly (dI·dC)–(dI·dC) as bulk carrier DNA, 2.5 μg bovine serum albumin, radioactive DNA probe (50 000

c.p.m.) and IclR protein. After 10 min of incubation at 25°C, 10 μl of the above mixture were loaded onto a 4% polyacrylamide gel at low ionic strength and electrophoresed for 2 h at 10 V/cm. Radioactive compounds were detected by autoradiography after overnight exposure to Amersham MP film at −70°C.

DNase footprinting

The footprinting method of Galas and Schmitz (1978) using bovine pancreas deoxyribonuclease I (grade I, Boehringer Mannheim) was utilized. After 10 min of incubation at 25°C of the IclR protein and the end-labeled DNA probe in binding buffer, the mixture was treated for 30 s at the same temperature with 15 ng DNase I dissolved in 5.5 mM $CaCl_2$ and 55 mM $MgCl_2$. The DNase digestion was stopped by addition of 20 mM EDTA and the sample was electrophoresed as described above.

After electrophoresis the bands corresponding to free and complexed DNA were visualized by autoradiography. Labeled DNA was then electroeluted, precipitated by ethanol, washed with 70% ethanol and dried. Samples were dissolved in sequencing sample buffer and electrophoresed in a sequencing polyacrylamide field gradient gel (4%) (Maniatis *et al.*, 1982).

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