

The HsdS polypeptide of the Type IC restriction enzyme *EcoR124* is a sequence-specific DNA-binding protein

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Summary

The HsdS and HsdM polypeptides of the type IC restriction enzyme *EcoR124* have been purified independently and used in a set of gel retardation experiments to determine the minimum requirements for sequence-specific recognition of DNA by this enzyme. The HsdS polypeptide alone is able to bind to DNA in a sequence-specific manner. In addition, whilst the presence of the HsdM polypeptide gives rise to a stimulation of DNA binding by the HsdS subunit it is not clear whether, under the conditions of the experiments reported here, the HsdS subunit maintains the same interactions with the HsdM subunits observed in the absence of DNA.

Introduction

A recent review of restriction and modification systems (Wilson and Murray, 1991) highlights the imbalance in our knowledge of the properties of complex (Types I and III) restriction and modification enzymes compared with the Type II enzymes which are invaluable reagents in experimental molecular biology. Nevertheless, a substantial body of knowledge has been accumulated concerning the genetics and enzymology of a small number of Type I and III restriction enzymes (see Bickle, 1987, and references therein). The Type IC enzyme (Bickle, 1987) *EcoR124* is the product of three genes: *hsdS*, *hsdM* and *hsdR* encoding three polypeptides which constitute an active restriction enzyme complex (Firman *et al.*, 1985; Price *et al.*, 1987; 1989). The stoichiometry of the restriction endonuclease complex has not been unequivocally established, but the HsdS and HsdM polypeptides assemble as a trimer comprising a single HsdS subunit and two HsdM subunits to produce a DNA sequence-specific adenine methyltransferase (DNA Metase) (Taylor *et al.*, 1992).

Recently, a number of publications have discussed the relationship between the HsdS polypeptides of the allelic restriction and modification systems *EcoR124* and *EcoR124/3*. These two enzymes differ only with respect to the number of repeats of a protein sequence motif (TAEI) in the primary sequence of the HsdS polypeptide (Price *et al.*, 1989; Gubler and Bickle, 1991; Gubler *et al.*, 1992). It appears that this motif, which has been found in all of the known Type IC HsdS polypeptides, may be involved in the spatial orientation of the two sequence-specific DNA-binding domains which are characteristic of all Type I restriction enzymes (Gubler and Bickle, 1991; Gubler *et al.*, 1992). However, despite a series of elegant genetic experiments which defined the function of the *hsd* genes of Type I systems (reviewed by Bickle, 1982; Cowan *et al.*, 1989), it has not been formally demonstrated that the HsdS polypeptide alone is responsible for sequence-specific DNA recognition. In this paper we report the subcloning and high-level expression of the *hsdS* gene of the *EcoR124* restriction and modification system using the glutathione-S-transferase (GST) fusion vector, pGEX-2T. The purified HsdS polypeptide shows sequence-specific DNA recognition of an oligodeoxynucleotide containing the recognition sequence, GAA(N)₆ATCG.

Results

High-level expression and purification of the *EcoR124* HsdS and HsdM polypeptides

The plasmid pUNG20 (Firman *et al.*, 1985) was used as the template in polymerase chain reactions (PCRs) using primers tailored for subcloning the coding sequences of the *EcoR124* *hsdS* and *hsdM* genes into the *Sma*I and *Bam*HI/*Eco*RI sites of pGEX-2T respectively (see Fig. 1). This strategy yielded high-level expression of two soluble fusion proteins which we have called GST/HsdS and GST/HsdM following induction of *Escherichia coli* Ru308 with IPTG. In order to compare the properties of the individual subunits with the wild-type enzyme, *E. coli* AB2463 harbouring the *EcoR124* *hsdS* and *hsdM* genes expressed from their natural promoter was used as a source of the *EcoR124* Metase.

Purification of the fusion proteins GST/HsdS and GST/HsdM was achieved by single-step chromatography on

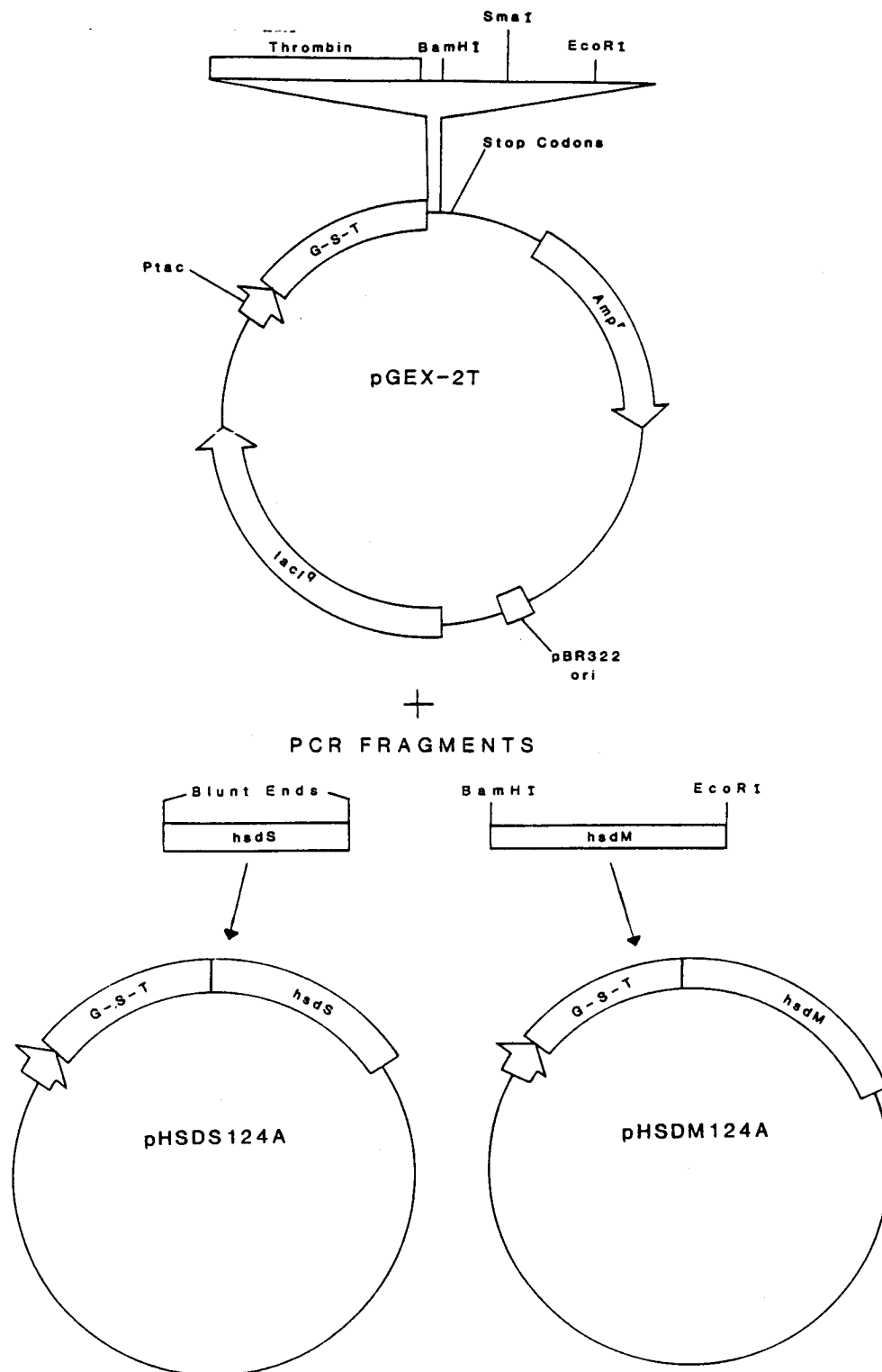


Fig. 1. Strategy used to subclone the EcoR124 *hsdS* and *hsdM* genes into the glutathione-S-transferase vector, pGEX-2T.

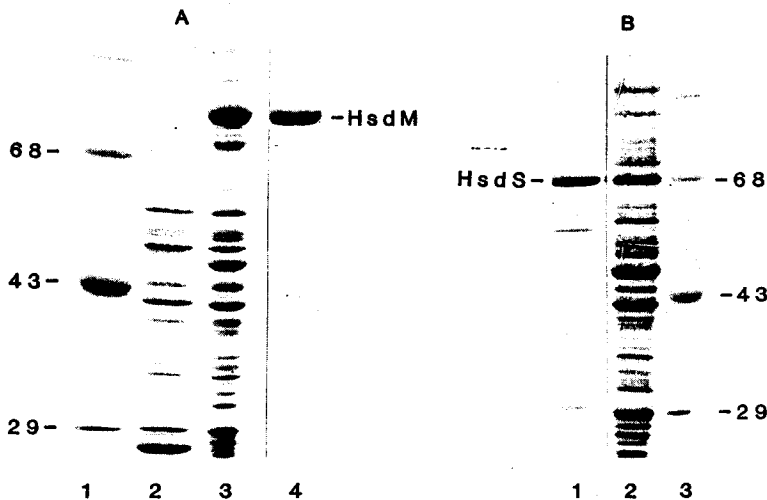


Fig. 2. A. SDS-PAGE of the GST/HsdS fusion protein. Lanes: 1, molecular weight standards; 2, crude extract of *E. coli* Ru308 harbouring pGEX-2T; 3, crude extract of *E. coli* Ru308 harbouring pHSDM124A; 4, purified GST/HsdM following affinity chromatography on glutathione agarose. B. SDS-PAGE of the GST/HsdS protein. Lanes: 1, purified GST/HsdS fusion protein; 2, crude extract of *E. coli* Ru308 harbouring pHSDS124A following induction with IPTG; 3, molecular weight standards.

glutathione agarose (Fig. 2). Experiments with the *EcoR124* Metase were carried out using crude extracts of *E. coli* AB2463, without further purification.

Sequence-specific DNA recognition by the *EcoR124* HsdS polypeptide

Using gel retardation of oligodeoxynucleotide duplexes containing the *EcoR124* recognition sequence, it has been shown by Taylor *et al.* (1992) that purified *EcoR124* Metase binds to its recognition sequence with an affinity of the order 10^8 M^{-1} . In addition, the affinity of the protein for non-cognate oligodeoxynucleotide duplexes is two orders of magnitude lower. In lane 1 of Fig. 3A, the complex formed between a cognate oligodeoxynucleotide duplex and *EcoR124* Metase migrates to the same position as that in lane 2, in which purified HsdS protein has been incubated with the same DNA duplex. In this experiment,

the GST/HsdS fusion protein has been cleaved with the protease thrombin to release the HsdS polypeptide.

In order to determine the effect of the HsdM polypeptide on sequence-specific DNA binding by the HsdS subunit, the HsdS fusion protein before and after thrombin cleavage was incubated with the HsdM subunit in the presence of the cognate oligodeoxynucleotide duplex. Lane 1 of Fig. 3B shows the HsdS:DNA complex, and in lanes 2 and 3 are the results of addition of the HsdM polypeptide (prepared by thrombin cleavage of the purified GST/HsdM fusion protein) to GST/HsdS and the free HsdS polypeptide. It is clear that the addition of the HsdM subunit either does not enhance the affinity of the specificity subunit for DNA or has a relatively minor effect, but confirms that the complex does not alter its position in the retardation gel. Moreover, whilst there is a band arising from the interaction of the GST/HsdS fusion protein with DNA, this is substantially weaker than the interaction

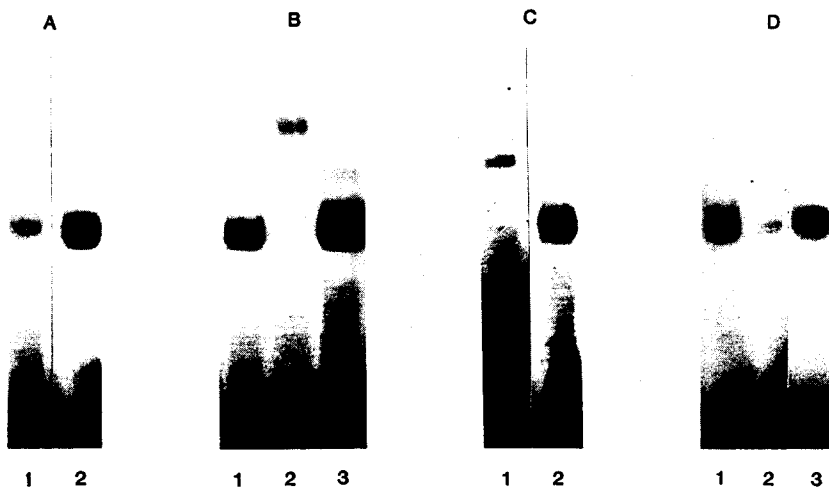


Fig. 3. A. Gel retardation of a cognate oligodeoxynucleotide duplex by *EcoR124* DNA Metase (lane 1) and purified HsdS following cleavage of GST/HsdS with thrombin (lane 2). B. The effect of HsdM on DNA binding by HsdS. Lanes: 1, HsdS following thrombin cleavage of the fusion protein; 2, addition of the GST/HsdS fusion to cognate DNA without prior addition of thrombin; 3, as lane 1 but in the presence of the nascent HsdM polypeptide. C. Sequence-specific binding by HsdS. Lanes: 1, HsdS and a non-cognate oligodeoxynucleotide; 2, HsdS binding to a cognate oligodeoxynucleotide duplex. Equivalent amounts of protein and DNA were used in each incubation. D. Competition assays between the HsdS polypeptide in complex with a cognate oligodeoxynucleotide duplex (lane 1), unlabelled cognate duplex at a 10-fold excess (lane 2), and non-cognate DNA at a 100-fold excess (lane 3).

between the nascent specificity polypeptide and the oligodeoxynucleotide duplex. In a separate experiment (results not shown), the addition of the HsdM fusion protein to nascent HsdS did not result in a retardation of the HsdS:DNA complex. This latter observation is consistent with either a reduction in the affinity of HsdM for HsdS in the presence of a cognate DNA duplex or impaired function of the HsdM subunits when constrained by the presence of glutathione-S-transferase.

Contribution of the HsdM polypeptide to sequence-specific DNA recognition by the specificity subunit

Since the specificity subunit of *EcoR124* (and indeed *EcoK*; D. T. Dryden and N. Murray, personal communication) associates with two HsdM subunits in solution to form a functional DNA methyltransferase (Taylor *et al.*, 1992), it is perhaps somewhat surprising that the addition of the HsdM polypeptide does not retard the mobility of the HsdS:DNA complex in the experiments shown in Fig. 3B. That the complex is sequence specific is demonstrated by both the result shown in Fig. 3C, in which equivalent amounts of a specific and a non-specific oligodeoxynucleotide duplex were incubated with the purified HsdS polypeptide, and competition experiments in which the labelled DNA duplex is challenged by an unlabelled non-cognate DNA duplex (see Fig. 3D). The slower migrating, weak band in Fig. 3C, lane 1 is a non-specific complex probably between at least two HsdS polypeptides and the DNA duplex and was only obtained by prolonged exposure of the gel. Similar results with a non-cognate DNA duplex were reported by Taylor *et al.* (1992) when an excess of the *EcoR124* Metase was added to a non-cognate oligodeoxynucleotide.

Following a gel retardation experiment similar to that described above (see Fig. 3B, lane 3), the nucleoprotein complex was excised from the gel and re-electrophoresed on a continuous SDS-polyacrylamide gel (see the *Experimental procedures*). From the results of silver-staining (see Fig. 4), it is clear that the HsdM polypeptide in the presence of the cognate oligodeoxynucleotide duplex migrates at the same position as the HsdS:DNA complex. However, in order to determine whether the HsdM subunit is part of a complex, the HsdM polypeptide alone was subjected to the same procedure in the absence of DNA. In order to visualize the HsdM polypeptide an excess of this species was added over the HsdS polypeptide. In Fig. 4, lanes 2 and 3 show that the HsdM polypeptide can be detected migrating at the same position in the first electrophoresis experiment in the presence (lane 3) or absence (lane 2) of HsdS. Indeed, both lanes appear to contain equivalent amounts of the HsdM polypeptide and therefore it is not clear whether under the experimental conditions used here the two components of the *EcoR124*

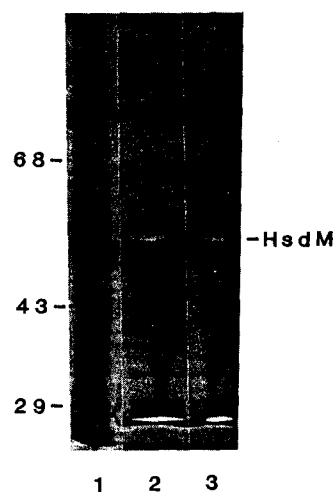


Fig. 4. Elution of the HsdM polypeptide following gel retardation experiments. Lanes: 1, molecular weight standards; 2, HsdM eluted from a retardation experiment in which HsdM was incubated with a cognate DNA duplex in the absence of HsdS; lane 3, as in lane 2 but including HsdS in the initial incubation.

Metase are indeed simply comigrating fortuitously or are in association with each other and the DNA duplex. Nevertheless, it is clear that the specificity subunit alone is responsible for sequence-specific DNA recognition and that the effect of the presence of the HsdM polypeptide is minimal. Therefore, it is possible that the interactions between HsdS and HsdM in the absence of DNA are different from those in the presence of DNA.

Discussion

The functions of the separate subunit species of complex Type I restriction endonucleases have been inferred from a combination of genetic and biochemical studies (see Bickle, 1982, for a review of the early literature). However, it has never been clear whether sequence-specific DNA recognition is mediated by the specificity subunit alone or in conjunction with the methyltransferase subunit. Genetic evidence supports the view that the HsdS subunit is an independent DNA-recognition subunit, since all mutants that have lost the capacity to bind to DNA map to the *hdsS* gene. However, a recent examination of mutants of *EcoK* which show altered discrimination between hemimethylated and unmethylated DNA suggests a role for the HsdM polypeptide in protein-DNA interactions (Kelleher *et al.*, 1991). In order to clarify this issue we have expressed and purified the separate specificity and methyltransferase components of the Type IC restriction enzyme, *EcoR124*. The use of the glutathione-S-transferase fusion vector pGEX-2T facilitated high-level soluble expression and subsequent purification of the two polypeptide species.

It has been demonstrated that the HsdS polypeptide alone interacts with DNA in a sequence-specific manner and that, somewhat surprisingly, the HsdM polypeptide has only a minor stimulatory effect on this interaction, as judged by gel retardation experiments. Since it is known that the EcoR124 Metase (Taylor *et al.*, 1992) and EcoK Metase exist as trimers in solution in the absence of DNA, and that the addition of DNA to EcoK, for example (D. Dryden, L. Powell and N. Murray, personal communication), does not lead to dissociation of the complex, we can only assume that this EcoR124 complex is not stable under the conditions of the retardation experiments reported here. One other factor which may influence the association of the HsdM subunit with the HsdS:DNA complex is the length of the DNA duplex. The oligonucleotide used in these experiments is 25 bp in length whereas that used by Taylor *et al.* was a 30-mer. It may be that subunit interactions within the ligand-free EcoR124 Metase are different from those within the binary complex between the enzyme and DNA. For example, an exchange of protein:protein contacts for protein:nucleic acid contacts between the bases which flank the recognition elements and the HsdM subunits may occur during substrate addition to the enzyme. Indeed it is apparent that the type II DNA Metases, M.EcoRI (Reich *et al.*, 1991) and M2.HgaI (G. S. Baldwin and D. P. Hornby, unpublished), undergo significant conformational changes during the formation of catalytic intermediates. Therefore, whilst the HsdS polypeptide does indeed form a sequence-specific complex with a 25 bp duplex containing the EcoR124 site, a detailed investigation of the interactions between this subunit in the presence and absence of the HsdM polypeptide and larger DNA duplexes by footprinting studies should determine whether the HsdS interactions are modulated by catalytic events. Moreover, such experiments should reveal whether the formation of a stable EcoR124 binary complex with DNA requires a DNA duplex greater in length than the one used here.

Experimental procedures

Bacterial strains and plasmids

E. coli Ru308, which reportedly suppresses the formation of inclusion bodies (A. Bannister, personal communication), was used as a transformation host for the expression constructs pHSDS124A and pHSDM124A and was a gift from Dr A. Bannister (Dept of Pathology, University of Cambridge, England). The *E. coli* K-12 strain AB2463, harbouring the plasmid pJS62, a derivative of pJS54 (Gubler and Bickle, 1991) encoding the EcoR124 *hsdS* and *hsdM* genes inserted in the *PvuI*-*Hin*dIII sites of the kanamycin gene, was used as a source of the wild-type enzyme. The plasmid pUNG20 (Firman *et al.*, 1985) was used as a

template for all PCRs. The fusion vector pGEX-2T was purchased from Pharmacia.

Enzymes and chemicals

All restriction enzymes, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from Northumbria Biologicals Ltd, Boehringer Mannheim or New England Biolabs. Taq DNA polymerase was from Promega, deoxynucleoside triphosphates were from Pharmacia, and radiolabelled compounds were from Amersham International. All other chemical reagents were from Sigma, BDH or Gibco.

DNA manipulations

Engineering of the constructs pHSDS124A and pHSDM124A was by use of the PCR. Following restriction digestion and purification of the PCR fragments carrying the genes encoding the HsdS and HsdM subunits, they were ligated into the *Sma*I and *Bam*HI-EcoRI sites of the fusion vector pGEX-2T, respectively, using standard DNA-manipulation procedures.

Transformation of *E. coli* strain Ru308 with the constructs was carried out using the calcium chloride/rubidium chloride procedure of Kushner (1978).

Protein expression and purification

Induction of fusion protein expression was by the addition of 20 $\mu\text{g ml}^{-1}$ IPTG to a 12.5–25-fold dilution of an overnight culture of Ru308 cells harbouring either pHSDS124A or pHSDM124A. Cultures were incubated for 4 h prior to addition of IPTG and were then induced for a further 3 h, with all incubations being carried out at 30°C. Cells were harvested by centrifugation at 6000 $\times g$ for 10 min at 4°C and, after sonication in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8), the supernatant containing the soluble fusion protein was separated from the remainder of the cell proteins by centrifugation at 15000 $\times g$ for 20 min at 4°C.

Purification of the fusion protein was by glutathione-affinity chromatography. The supernatant was loaded onto a 10 ml glutathione-agarose column pre-equilibrated with PBS (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄ pH 7.3, 1% Triton X-100) and left at room temperature for 10 min before washing with 10 column volumes of PBS. The fusion protein was eluted with 50 mM Tris-HCl pH 8 containing 5 mM reduced glutathione.

Electrophoresis

Crude and purified cell extracts of fusion proteins GST/HsdS and GST/HsdM were analysed by SDS-PAGE on 9% gels run in glycine buffer (24.67 mM Tris-HCl pH 8.8, 3.47 mM SDS, 191.82 mM glycine) and stained with Kenacid blue R (milling blue 2BR reagent) by the method of Laemmli (1970).

Gel fragments containing nucleoprotein complexes were excised from 'retardation assay' gels (see below) and run on an SDS-polyacrylamide gel as above, with protein species being visualized by silver staining.

Non-denaturing polyacrylamide gels were used for the band-shift assay. Briefly, after pre-incubation for 3 min at room temperature in the absence of 'labelled probe', oligodeoxynucleotide duplexes labelled using a [α -³²P]-dATP were incubated

for a further 30 min in the presence of non-specific competitor DNA (poly dI-dC), binding buffer (10mM Tris-HCl pH 7.5, 50mM KCl, 5mM MgCl₂, 1mM dithiothreitol, 12.5% glycerol, 0.1% Nonidet P40, 1mM EDTA) and protein extract and run on 4% gels in TBE buffer (44.5mM Tris-borate, 44.5mM boric acid, 1mM EDTA pH 8). Protein-DNA complexes formed were visualized by exposing the dried gels to X-ray film at -70°C using film cassettes containing intensifying screens.

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