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Lac repressor with the helix – turn – helix motif of λ cro binds to *lac* operator

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Lac repressor, λ cro protein and their operator complexes are structurally, biochemically and genetically well analysed. Both proteins contain a helix – turn – helix (HTH) motif which they use to bind specifically to their operators. The DNA sequences 5′-GTGA-3′ and 5′-TCAC-3′ recognized in palindromic *lac* operator are the same as in λ operator but their order is inverted from head to head to tail to tail. Different modes of aggregation of the monomers of the two proteins determine the different arrangements of the HTH motifs. Here we show that the HTH motif of λ cro protein can replace the HTH motif of Lac repressor without changing its specificity. Such hybrid Lac repressor is unstable. It binds *in vitro* more weakly than Lac repressor but with the same specificity to ideal *lac* operator. It does not bind to consensus λ operator.

Key words: HTH motif/Lac repressor/λ cro/λ repressor/protein – DNA interaction

Introduction

Lac repressor and λ repressor are paradigms of negative control of transcription (Jacob and Monod, 1961). Both systems have been studied extensively with regard to specific DNA recognition. They both use helix – turn – helix (HTH) motifs to recognize DNA (Matthews *et al.*, 1982). Their recognition helices cross the major groove of DNA almost parallel to the base pairs in both complexes. The HTH motifs of λ repressor (Jordan and Pabo, 1988), λ cro protein (Brennan *et al.*, 1990) and of Lac repressor (Lamerichs *et al.*, 1989) are able to recognize specifically the same DNA motif 5′-GTGA-3′ or 5′-TCAC-3′ (Figure 1). The same two residues of their recognition helices evidently recognize with different side chains the same three base pairs (Boelens *et al.*, 1987; Lehming *et al.*, 1988; Lamerichs *et al.*, 1989). NMR studies (Boelens *et al.*, 1987; Lamerichs *et al.*, 1989) and genetic analysis (Lehming *et al.*, 1988) suggested that the HTH motifs of these lambdoid and bacterial repressors are arranged in opposite orientations.

Figure 1 shows the recognition units of Lac repressor and λ cro protein. We define a recognition unit to consist of the HTH motif of one protein monomer and one half-operator. To emphasize that both proteins possess similar recognition units, the central recognition unit is drawn such that it is shared by Lac repressor and λ cro protein in the two dimensional representation of Figure 1. The dimerization interface of the two λ cro monomers lies on the left side of the shared recognition unit, while the dimerization interface of the two Lac repressor monomers lies on its right side. This model predicts that the HTH motifs of the two proteins could be exchanged and that the dimeric structure of the host protein will determine which target will be recognized. Thus, the HTH motif of λ cro transplanted into Lac repressor should specifically bind to ideal *lac* operator, but no longer to consensus λ operator. We constructed such a hybrid Lac repressor and found it was indeed able to bind to *lac* operator but not to consensus λ operator.

Results

Construction and purification of hybrid Lac repressors

We constructed two hybrid *lacI* genes coding for tetrameric Lac repressors with HTH motifs of the λ cro protein. One construct contains the complete HTH motif i.e. residues 16 – 35 of the λ cro protein. They replace residues 6 – 25, the HTH motif of Lac repressor. In the other modified hybrid repressor we exchanged only those residues of the HTH motif which are exposed on the surface and may come close to the DNA upon binding. This modified hybrid gene was constructed because NMR studies of Kaptein's group (Kaptein *et al.*, 1985) have revealed specific intramolecular contacts between residues 6, 8, 9, 12, 13, 20, 23 and 24 (Figure 2a) and residues of helix III of Lac repressor. In this second construct Leu6, Asp8, Val9, Tyr12, Ala13, Val20, Val23 and Val24 of Lac repressor were not exchanged for the corresponding residues of the λ cro protein since some of their contacts with residues of the following helix III might have been indispensable to maintain the overall tertiary structure of the HTH domain and its correct package into the headpiece or the whole protein. In all cases the choice of residues which were not exchanged relies on the NMR studies only. Figure 2a compares the amino acid sequences of the HTH motifs of Lac repressor, of λ cro as inserted into the hybrid Lac [HTH λ cro] repressor and of the modified Lac [HTH λ cro] repressor. Note that only three residues are identical in the *lac* and λ cro HTH motifs (Matthews *et al.*, 1982) and that the modified Lac [HTH λ cro] repressor possesses all those amino acids of λ cro which are involved in specific contacts to the base pairs of the operator DNA (Brennan *et al.*, 1990).

We anticipated that inevitable deviations in the molecular architectures of the hybrid Lac repressors would impair the exact positioning of the recognition helices. This would diminish or perhaps even abolish the function of the λ cro HTH motif embedded in the Lac repressor framework. We therefore started from the tight binding X86 Lac repressor mutant and introduced both the complete and the modified HTH motif of λ cro into the X86 background. The X86 Lac

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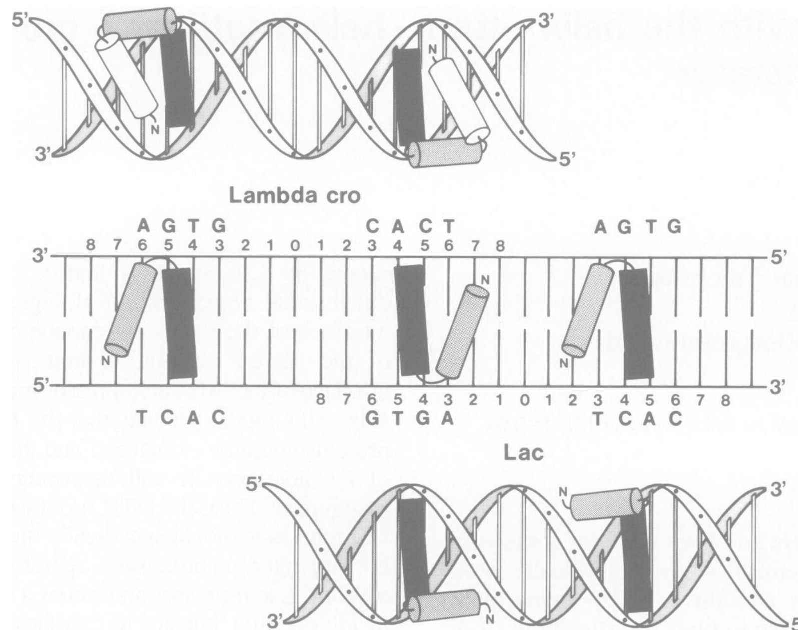

Fig. 1. Schematic representation of the operator complexes of λ cro protein and Lac repressor. The two dimensional model illustrates how the dimerization interfaces of the repressors determine the orientation of the two HTH motifs towards each other. Note that the upper strand is written from 3′ to 5′ to allow the recognition helices to lie in front of the double helix. The recognition helices are depicted in black, the helices preceding the recognition helices in grey. In the case of λ cro protein, the first helix of the protein which precedes the HTH motif is depicted in white. There is no corresponding helix in Lac repressor. The *lac* operator shown here carries a central base pair like natural *lac* operator. Ideal *lac* operator (Simons *et al.*, 1984) lacks this central base pair. Only those base pairs which are presumably recognized by the recognition helices are indicated. We do not show the specific interactions between amino acid side chains of the recognition helices and base pairs. The X-ray analysis of the cocrystal of λ cro protein and its operator (Brennan *et al.*, 1990) does not unequivocally resolve the specific contacts between the second (serine) and the sixth (lysine) amino acids of the recognition helix and base pairs 3−6 (5′-TCAC-3′) of the λ cro consensus operator [the base pairs are numbered from the centre of symmetry of the operator as proposed by Lehming *et al.* (1987); the single central base pair being numbered 0]. Serine 2 (28), the second residue of the recognition helix and residue 28 of λ cro protein, comes close to base pairs 5 and 6. We presume that it contacts guanine 5 and adenine 6 of λ cro consensus operator. Lysine 6 (32) recognizes base pairs 4 and 5 and we presume that it contacts thymine 4 and guanine 5 respectively of the λ cro consensus operator (Brennan *et al.*, 1990). These assumptions are consistent with earlier genetic and structural studies (Hochschild and Ptashne, 1986; Hochschild *et al.*, 1986; Takeda *et al.*, 1989; Benson and Youderian, 1989). The corresponding amino acids of the recognition helix of Lac repressor, glutamine 2 (18) and arginine 6 (22) contact the 5′-GTGA-3′ by interacting similarly with the guanine of base pair 4 and the guanine of base pair 6 of *lac* operator respectively (Lehming *et al.*, 1987, 1988, 1990; Lamerichs *et al.*, 1989; Sartorius *et al.*, 1991).

repressor carries a serine to leucine exchange at position 61 far outside the HTH motif. This mutation increases *in vitro* DNA binding of Lac repressor ~30-fold (Jobe and Bourgeois, 1972; Pfahl, 1976; Schmitz *et al.*, 1978). The *in vivo* properties of the X86 mutant have been studied thoroughly and will be reported elsewhere (P.Kolkhof, in preparation). We also constructed a hybrid *lacI* gene with the HTH motif of λ repressor but this protein was totally degraded as judged by SDS gel analysis (data not shown) and therefore we did not pursue this further.

Both hybrid Lac [HTH λ cro] repressors turned out to be protease sensitive but the use of the protease inhibitor PMSF reduces greatly the degradation of the hybrid proteins during the first steps of protein enrichment. Unfortunately, this protease inhibitor was not able to reduce proteolysis during a further purification on phosphocellulose columns. Figure 2b shows a purification protocol of X86 Lac repressor and both hybrid Lac [HTH λ cro] repressors. All extracts or purified proteins shown in Figure 2b were prepared in the absence of any protease inhibitor. Lanes a, d and g show crude extracts immediately after sonification. Here no predominant band corresponding to Lac repressor can be seen. Thus it is not possible to judge the extent of degradation of the hybrid proteins in the crude extracts. Lanes b, e and h show the respective enriched crude extracts after precipitation with 30% $(NH_4)_2SO_4$. The predominant bands in each case represent Lac repressor or one of its degradation products. In the enriched crude extract (lane b) and after purification (lane c) wild-type X86 Lac repressor appears as single bands, whereas both hybrid Lac repressors show an additional faster migrating band below the proper band of intact repressor and smear in the running direction of the gel. These are products of N-terminal proteolysis which are observed in many such mutants (Schlotmann and Beyreuther, 1979). Surprisingly, the modified hybrid Lac [HTH λ cro] repressor turned out to be more sensitive to proteolytic breakdown than the hybrid repressor with the complete HTH motif of λ cro (compare the upper bands at the level of intact repressor in lanes e and h). Thus, the gain of stability expected for the modified hybrid repressor was not obtained, on the contrary this protein was even less stable. In the fractions after phosphocellulose chromatography (lanes f and i) the respective bands of the intact repressors were further diminished.

In vitro binding studies

We used enriched extracts of both hybrid repressors (see Materials and methods) to test their activity in DNase I protection and gel retardation experiments. Since the modified hybrid Lac [HTH λ cro] repressor showed no higher affinity for ideal *lac* operator in DNase I protection experiments (data not shown), presumably a consequence

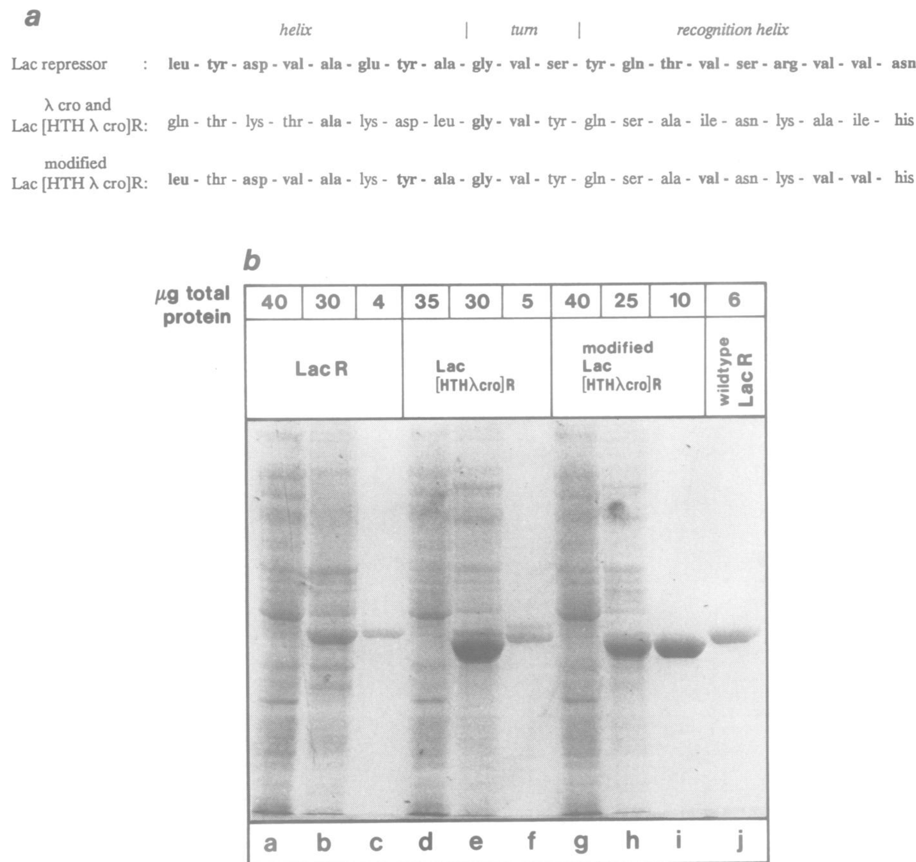

Fig. 2. (**a**) Amino acid sequence of the HTH motifs of Lac repressor, of λ cro as it is present in the hybrid Lac [HTH λ cro] repressor and in the modified hybrid Lac [HTH λ cro] repressor. The residues of Lac repressor, which are identical in the other two HTH motifs are indicated in bold face. The HTH motif of Lac repressor contains residues 6−25. (**b**) 10% SDS gel with samples of tetrameric X86 Lac repressor, the tetrameric hybrid X86 Lac [HTH λ cro] repressor and the tetrameric modified hybrid X86 Lac [HTH λ cro] repressor after various stages of purification (lanes a−i). The gel was stained with Coomassie blue. Lanes a, d and g are crude extracts mixed with sample buffer immediately after sonification. Lanes b, e and h are enriched crude extracts obtained by precipitation from the crude extracts with 30% $(NH_4)_2SO_4$. Lanes c, f and i show protein fractions after further purification by affinity chromatography with phosphocellulose columns. The marker protein in lane j is purified wild-type tetrameric Lac repressor. The protein extracts shown here were not treated with the protease inhibitor PMSF (see Materials and methods) during their enrichment and further purification. The preparation of an enriched crude extract of the tetrameric hybrid Lac [HTH λ cro] repressor in the presence of PMSF produces a protein fraction which is at least 50-fold more active in DNA binding. According to [^{14}C]IPTG equilibrium dialysis (Miller, 1972) the fractions in lanes d (tetrameric hybrid Lac [HTH λ cro] repressor) and g (tetrameric modified hybrid Lac [HTH λ cro] repressor) contain 81% and 50% Lac repressor core molecules as compared with tetrameric wild-type Lac repressor (lane a).

of its higher protease sensitivity, we only used the hybrid Lac [HTH λ cro] repressor for further detailed analysis concerning its specificity in DNA binding.

We observed specific protection of ideal *lac* operator (Figure 3a). We titrated ideal *lac* operator with increasing amounts of the enriched crude extract to determine the specific binding capacity of wild-type and hybrid Lac [HTH λ cro] repressor extracts. A comparable protection of ideal *lac* operator was achieved by 100 ng extract with wild-type Lac repressor and 800 ng extract containing the hybrid Lac [HTH λ cro] repressor. In order to show that the observed DNase I protection is specifically caused by the hybrid Lac [HTH λ cro] repressor we added IPTG, an inducer of Lac repressor, to one sample (Figure 3a, lane h). In the presence of IPTG protection with extracts containing the hybrid Lac [HTH λ cro] repressor was significantly reduced. Wild-type λ cro protein (~50% purity) does not bind to ideal *lac* operator (Figure 3a). In the control experiment with two λ consensus operators (Figure 3b) we used 4 μg of the same enriched crude extract of wild-type Lac repressor and 4 μg of the enriched crude extract of the hybrid Lac [HTH λ cro] repressor. We see no protection of the two λ consensus operators which are protected by wild-type λ cro protein. Similar results are obtained in electrophoretic mobility shift assays (EMSAs), shown in Figure 4: protein extracts with the hybrid Lac [HTH λ cro] repressor retard DNA with ideal *lac* operator (Figure 4a, lane c) but not DNA with two λ consensus operators (Figure 4b).

We also analysed the dissociation rates of X86 Lac repressor and the hybrid Lac [HTH λ cro] repressor with ideal *lac* operator *in vitro* in gel shift experiments. The complex of X86 Lac repressor and ideal *lac* operator is exceedingly stable under normal conditions. We therefore used high salt concentrations for our kinetic dissociation experiments (300 mM KCl, 10 mM $MgCl_2$, 40 mM Tris−HCl pH 8.0, 0.1 mM DTT, 5% glycerol). We used a 100-fold excess of unlabelled ideal *lac* operator DNA to chase Lac repressor. Under these conditions the complex between dimeric X86 Lac repressor and ideal *lac* operator has a half life of ~90 min. The complex of tetrameric hybrid Lac [HTH λ cro] repressor and ideal *lac* operator is completely dissociated after 30 s (data not shown). The repressor−operator half-life does not depend on the quartenary structure of the repressor under these conditions

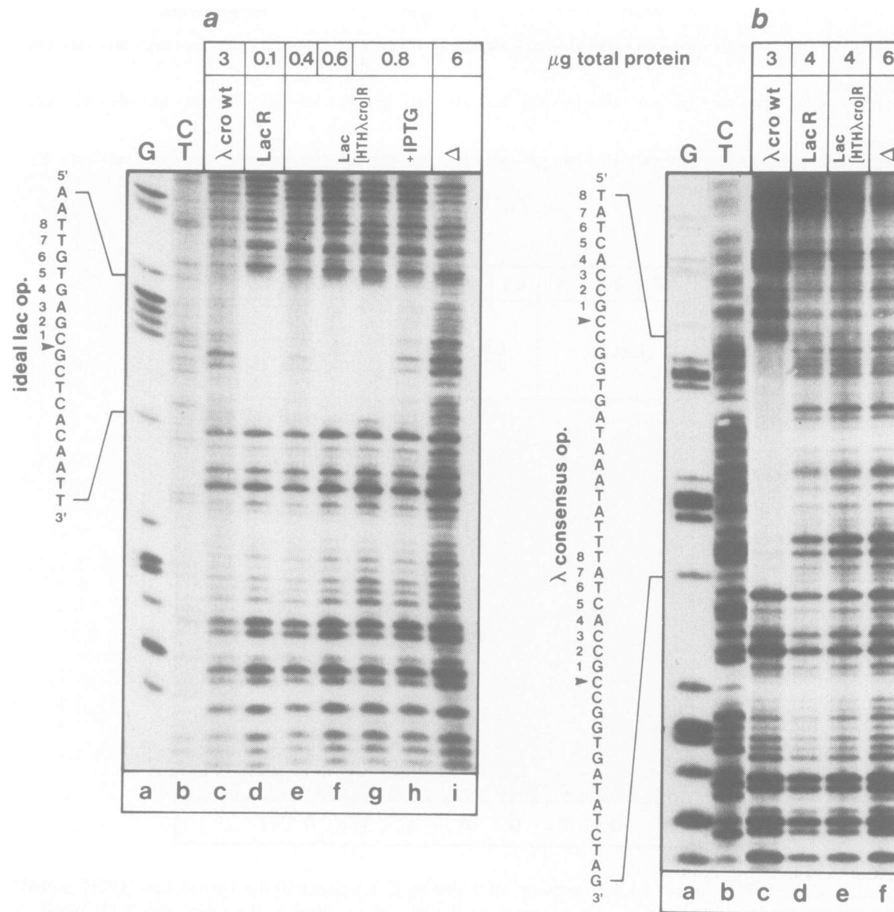

Fig. 3. DNase I protection experiments. (**a**) Specific binding of tetrameric X86 Lac repressor and tetrameric hybrid X86 Lac [HTH λ cro] repressor to ideal *lac* operator. (**b**) Control experiment with idealized symmetric λ consensus operators. Protein extracts containing tetrameric X86 Lac repressor or tetrameric hybrid X86 Lac [HTH λ cro] repressor, which fully protect ideal *lac* operator (**a**) do not protect the two λ consensus operators. 'Δ' indicates a control experiment in the presence of 6 μg protein precipitated by $(NH_4)_2SO_4$ from the same *E.coli* host lacking the plasmid. The wild-type λ cro protein used here was 50% pure (see Materials and methods).

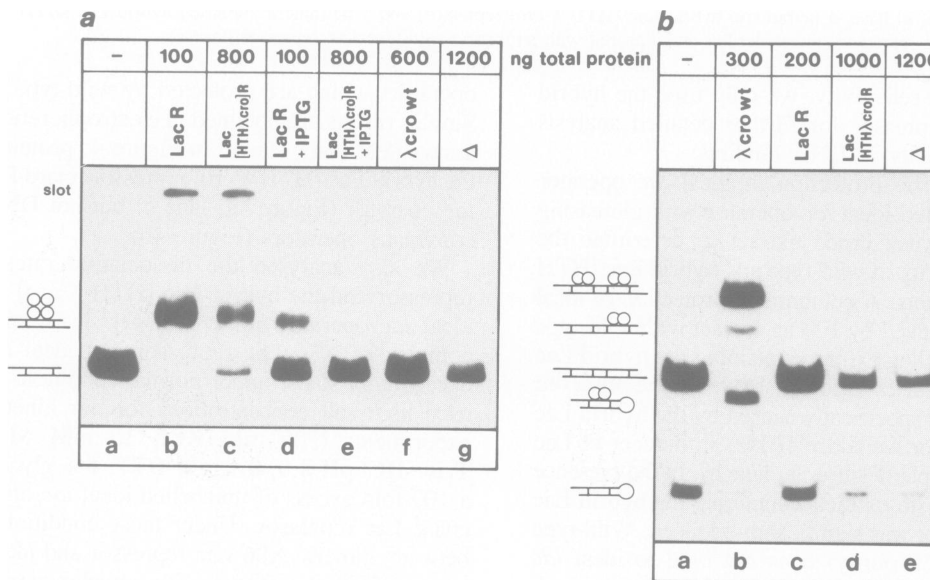

Fig. 4. Electrophoretic mobility shift assays (EMSAs). The symbols on the left sides of the figures indicate which structures or complexes cause the respective bands. (**a**) The enriched crude extracts described in Figure 3 retard a 152 DNA fragment containing ideal *lac* operator. (**b**) The same extracts do not retard a 46 bp DNA fragment containing two λ consensus operators. This fragment is well retarded by λ cro protein. The distance between the centres of symmetry of the two λ consensus operators is 23 bp (sequence see Figure 3). A single binding site is formed in the hairpin structure. Thus the hairpin can also be retarded by λ cro protein.

(Fickert and Müller-Hill, 1992). Thus the hybrid Lac repressor–operator complex is at least 200 times less stable than the corresponding complex with native Lac repressor.

The model illustrated in Figure 1 predicts critical contacts of the Lac [HTH λ cro] repressor to base pairs 4, 5 and 6 of *lac* operator. In order to test this prediction, we performed gel shift experiments with hybrid Lac [HTH λ cro] repressor and a *lac* operator variant which carries an adenine instead of a guanine in position 4 of *lac* operator (operator variant 41). The binding of hybrid Lac [HTH λ cro] repressor to this variant was drastically reduced as compared to ideal *lac* operator (Figure 5a). Moreover, we found no binding to a double operator variant with symmetric exchanges in positions 4 and 5 (Figure 5b). This indicates that particular specific contacts between the λ cro recognition helix and the operator DNA cannot be established when base pairs 4 and 5 are exchanged.

In order to exclude that the X86 mutation is responsible for the specific recognition of *lac* operator by Lac [HTH λ cro] X86 repressor, we tested X86 Lac repressor. We first constructed an X86 Lac repressor mutant with proline and lysine at positions 1 and 2 of the recognition helix. This mutant does indeed not bind to ideal *lac* operator (Lehming *et al.*, 1990) but to *lac* operator variant 4251 which carries a cytosine instead of a guanine in position 4 and an adenine instead of a thymine in position 5 (Figure 5b, lane j). Wild-type Lac repressor and Lac [HTH λ cro] repressor with the X86 exchange failed to retard this operator variant (Figure 5b, lanes k and l). We also determined the ratio of repression of *lacZ in vivo* with X86 Lac repressor and ideal *lac* operator and its 27 symmetric variants with single symmetric base pair exchanges. All the values of repression were ~3-fold higher than with wild-type Lac repressor (P.Kolkhof, in preparation). We thus conclude in contrast to some older reports (Jobe and Bourgeois, 1972; Schmitz *et al.*, 1978) that the X86 mutation does not significantly alter the specificity of recognition of Lac repressor, and only serves to increase the non-specific affinity of Lac repressor for DNA.

Model building based on the crystal structure of λ cro protein (Ohlendorf *et al.*, 1982) suggested specific contacts between glutamine 1 (27) and an adenine just 5′ of the 5′-TCAC-3′ motif. Although this base pair is not considered in our model for the function of the respective HTH motifs, we probed the relevance of the proposed contacts for the Lac [HTH λ cro] repressor: we replaced the G:C pair at position 2 of ideal *lac* operator by a T:A pair (operator variant 24). Figure 5b shows that the hybrid Lac [HTH λ cro] repressor has no affinity for this operator variant (lane c). Changing base pair 2 in *lac* operator may interfere with other contacts of residues outside the HTH motif. Alternatively Q_1 of the transplanted recognition helix may not be able to establish this additional contact because of the surrounding foreign protein architecture. The corresponding residue of Lac repressor Y_1 is very unlikely to contact base pair 2 (Kisters-Woike *et al.*, 1991).

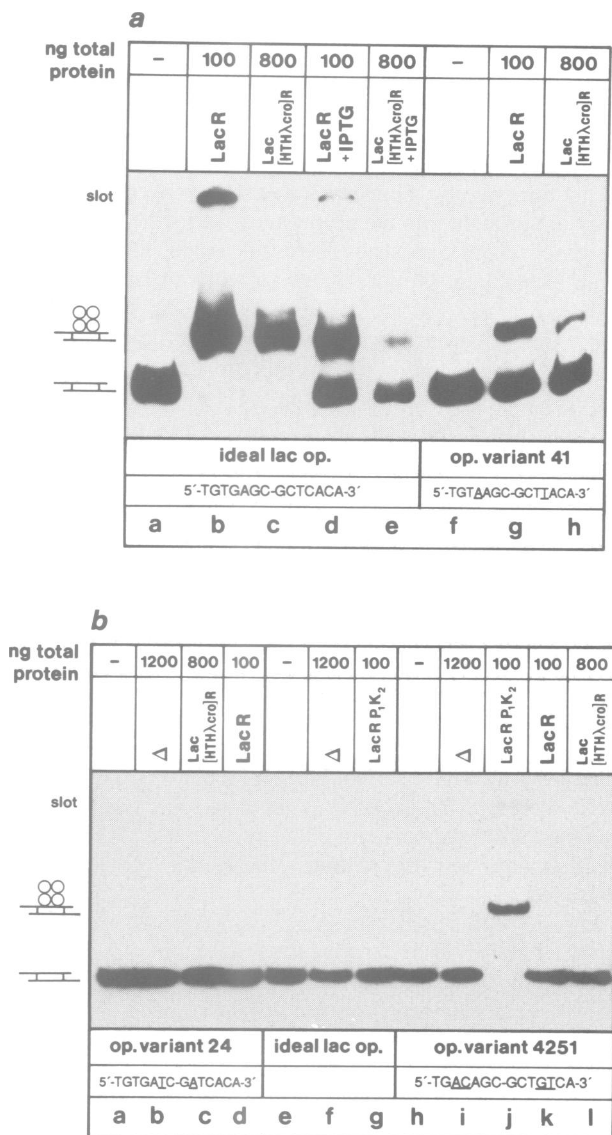

Fig. 5. Electrophoretic mobility shift assays (EMSAs). The symbols are the same as in Figure 4. The sequences of the different operator variants used in the experiments are depicted beneath the respective lanes. The underlined bases differ from ideal *lac* operator. (**a**) Enriched crude extracts of tetrameric hybrid X86 Lac [HTH λ cro] repressor as well as of tetrameric X86 Lac repressor retard the operator variant 41 to a much lesser extent than ideal *lac* operator. (**b**) The same enriched crude extract (Figure 3) shows little or no affinity to the operator variants 24 and 4251. The latter is well retarded by an enriched crude extract of tetrameric P_1K_2 X86 Lac repressor.

Discussion

The demonstration that the HTH motif of Lac repressor and the λ cro protein (and λ repressor) are oriented in opposite orientation is not new (Boelens *et al.*, 1987; Lehming *et al.*, 1988). Lehming *et al.* (1988) also pointed out that the recognition helices of both repressors recognize the same or very similar DNA sequences. If one considers that λ cro is made up from only 66 amino acids and that its HTH motif and the preceding helix consist of 35 amino acids one has to postulate that the remaining 31 residues are capable of dimerizing just by themselves. Inspection of the X-ray structure of λ cro (Anderson *et al.*, 1981) indicates that this is not the case. Some residues of the λ cro HTH motif help dimerization. If a foreign HTH motif were introduced into the λ cro protein some of the twenty residues are highly likely to disturb dimerization considerably. Such a disturbance was observed when only a few residues of the HTH motif of 434 repressor were swapped to 434 cro (Wharton *et al.*, 1984). The resulting hybrid 434 cro protein was unstable and rapidly degraded. In contrast to our experiment only those residues

which interact with DNA but not with the rest of the protein were exchanged.

In Lac repressor the situation is similar. Here the headpiece which is solely responsible for DNA recognition (Ogata and Gilbert, 1978) consists of only ~60 amino acids (Adler *et al.*, 1972). The two headpieces of dimeric Lac repressor do not dimerize by themselves (Geisler and Weber, 1976). They are brought into the proper position by the core of Lac repressor which consists of 300 amino acids. Thus the HTH swap experiment is more likely to work in Lac repressor than in λ cro.

We think that our *in vitro* evidence is sufficient to prove that the λ cro HTH motif functions in Lac repressor. But we note that it is evident that the HTH motif of λ cro does not fit properly into the context of Lac repressor headpiece. In fact the closely related experiment to introduce the HTH motif of λ repressor into Lac repressor failed (data not shown). Preliminary evidence indicates that the HTH motifs of Φ80 repressor and Φ80 cro function in the context of Lac repressor *in vitro* moderately well (data not shown).

Weakened operator binding and proteolysis are thus the main problems of HTH swap experiments. Proteolysis forced us to use enriched crude extracts of Lac [HTH λ cro] repressor almost immediately after sonication and precipitation. If we waited only a few hours the repressor activity decreased drastically. This made it impossible to purify the hybrid repressor further. It may be pointed out that λ cro itself (Pakula *et al.*, 1986) and some Lac repressor mutants with amino acid exchanges in the HTH motif (Schlotmann and Beyreuther, 1979) are proteolytically degraded.

In spite of this our experiments demonstrated clearly that *in vitro* Lac repressor carrying the HTH motif of λ cro binds specifically to *lac* operator and not to λ operator. But why do we see no *lac* repression *in vivo*? We need ~10 times more of the hybrid Lac [HTH λ cro] repressor than of wild-type Lac repressor for comparable gel shift or DNase protection. For gel shift and DNase protection intact dimers of Lac repressor are sufficient. We interpret the 10-fold difference in binding activity as indication that only 10% of dimers are intact in our protein samples, while the rest has been proteolytically degraded at the extreme N-terminus possibly also *in vivo*. Thus proteolysis may reduce repression up to 10-fold. We have further shown that the hybrid Lac [HTH λ cro] repressor binds *in vitro* at least 200 times less tightly to *lac* operator than the corresponding wild-type Lac repressor. Thus *in vivo* repression of Lac repressor will be reduced at least 2000-fold when the HTH motif of λ cro is introduced. The λ cro protein itself represses *in vivo* only 3- to 4-fold when P_R or P_{RM} with the natural λ operators are fused to *lacZ* (Hochschild and Ptashne, 1986). We had previously constructed variants of Lac repressor which carried the essential residues of λ cro in their recognition helices (Lehming *et al.*, 1990, 1991). The Lac repressor mutant Q_1S_2 represses the *lac* system *in vivo* ~20-fold (Lehming *et al.*, 1990). In contrast, various amino acid exchanges at positions 5 and 6 of Lac repressor recognition helix abolish binding to ideal or any other *lac* operator variant *in vivo*. This is true for the single exchanges S_5 to N_5 or R_6 to K_6 (as in λ cro) as well as for S_5 to G_5 or R_6 to A_6 (as in λ repressor). The inability of these mutants to bind *lac* operator *in vivo* may reflect small differences concerning the mode of penetration of the major groove by the *lac* and λ recognition helices or, possibly also be caused by proteolytic degradation (Sartorius, 1990). The Lac repressor mutants $Q_1S_2I_8$ and $Q_1S_2I_8H_9$ repress the *lac* system 17- and 5-fold respectively (Lehming, 1990). The K_6 mutation abolishes binding *in vivo* with one exception. The exchange of the wild-type tyrosine to histidine in position 1 of the Lac repressor recognition helix broadens its specificity, presumably by allowing for more flexibility in the turn region, such that the recognition helix gains some freedom to adjust itself within the major groove (Kisters-Woike *et al.*, 1991). The H_1K_6 double mutant represses the *lac* system 20-fold. Both mutants Q_1S_2 as well as H_1K_6 independently recognize several operator variants almost equally well which may indicate that λ cro is less specific for its target than Lac repressor (Lehming *et al.*, 1991). Taking all this into account the lack of *in vivo* repression is not astonishing. Only sensitive *in vitro* analyses such as footprint or gel retardation experiments are able to demonstrate the drastically weakened but specific DNA binding of the hybrid proteins.

Finally we would like to point out that the targets of c-Myc 5′-CAC-GTG-3′ (Blackwell *et al.*, 1990) and of CREB (cyclic AMP responsive element) 5′-TGAC-GTCA-3′ (Yamamoto *et al.*, 1988) can be seen as variants of the targets of λ repressor 5′-CACXXXXXGTG-3′. If we delete the innermost 5 bp (X) of λ operator we transform it into the c-Myc target. The CREB target is similar although not identical to the c-Myc target. The question may be raised whether c-Myc and CREB use recognition helices in a similar manner to Lac and λ repressor but with different spacing. Whereas in the Lac and λ operator–repressor complexes the recognition helices are 10 bp apart they would be 3 bp apart in comparable c-Myc and CREB complexes. Model building shows that such an arrangement is sterically possible. We would like to point out here that the two models for Jun/GCN4 target recognition (Vinson *et al.*, 1989; O'Neil *et al.*, 1990) are beautiful and possible but not proven by X-ray or NMR analysis. In fact the recent specificity change in the yeast bHLH protein CBF1 reported by Dang *et al.* (1992) seems to support our model: the position of the valine to arginine exchange would correspond to position 6 of the putative recognition helix of CBF1.

To sum up: it is most pleasing to see that Lac and λ, the two paradigmatic systems of bacterial gene regulation (Jacob and Monod, 1961) are so similar that one may say that the right side of one repressor corresponds to the left side of the other. Do they have a common ancester or have they arisen independently as mirror images? We have demonstrated here that the two possible head to head or tail to tail configurations of prokaryotic operators are recognized by repressors carrying HTH motifs in opposite orientation. So at last the question may be asked, does there exist the as yet missing HTH repressor–operator system which makes use of an operator with half-sites in head to tail configuration?

Materials and methods

Chemicals, enzymes and oligonucleotides

DNase I was obtained from Boehringer (Mannheim, Germany) and phosphocellulose was obtained from Bio-Rad. All other chemicals and enzymes are the same as listed in Lehming *et al.* (1987). The oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer. They

were purified on denaturating polyacrylamide gels (Sambrook *et al.*, 1989) prior to cloning and sequenced according to Sanger *et al.* (1977).

Bacterial strain, plasmids and constructs

Cells of *Escherichia coli* strain BMH 8117 [(*lac pro*)Δthi supE Nalr] were used as host for preparing enriched crude extracts and purified proteins. DNA coding for the HTH motifs of λ cro protein or the modified HTH motif of λ cro were cloned between the *Nde*I and *Xma*I sites in the *lacI* gene of pWB 1000 (Lehming *et al.*, 1988) using synthetic oligonucleotides (pWB 1000 codes for wild-type tetrameric Lac repressor). The resulting plasmids and pWB 1000 were used to introduce amino acid exchanges into the recognition helices of λ cro and Lac repressor respectively (for details of the cloning methods see Lehming *et al.*, 1987). In addition all repressor plasmids carry the serine to leucine exchange at codon 61 in the *lacI* gene (the X86 mutation), which increases non-specific DNA binding. The *lacI* gene in pWB 1000 was exchanged for the λ cro gene to purify the wild-type λ cro protein. The resulting plasmid was designated crosep. All operators used in the retardation assays and DNase I protection experiments were cloned into the *Xba*I site of pWB9, a plasmid derived from pWB300 (Lehming *et al.*, 1987) with a modified promoter region. All constructs were verified by DNA sequencing according to Sanger *et al.* (1977).

Preparation of enriched crude extracts and purified proteins

Cells of *E.coli* strain BMH 8117 carrying pWB 1000, derivates of it or no plasmid were resuspended in lysis buffer (1 g of cells per 3 ml of buffer: 50 mM Tris–HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.1 mM DTT). Cell lysis was performed with lysozyme as described by Sambrook *et al.* (1989) or by sonification. To reduce proteolysis 9 μl of 50 mM phenylmethylsulfonylfluoride (PMSF) were added every 20 min to 3 ml until storage at −70°C. After centrifugation $(NH_4)_2SO_4$ was added to the clear supernatant to a final concentration of 30%. The precipitated proteins were dissolved in KPG buffer (0.075 M K_2HPO_4:KH_2PO_4 5:1, 0.3 mM DTT, 0.1 mM EDTA, 0.15 mM PMSF, 5% glucose) and dialysed twice against the same buffer. They were stored at −70°C until they were used for DNase I protection (Galas and Schmitz, 1978; Simons *et al.*, 1984) and gel retardation experiments. For further purification, the dialysed material was loaded onto 10 ml phosphocellulose columns and eluted as described by Müller-Hill *et al.* (1971). The wild-type λ cro protein used in Figure 3a and b was purified by precipitation of crude extract of BMH 8117 cells containing the crosep plasmid with 90% $(NH_4)_2SO_4$. The precipitated protein was dialysed and purified by affinity chromatography on a phosphocellulose column and gel-filtration on a Sephadex G-75 column as described by Takeda *et al.* (1986). The λ cro preparation was ~50% pure as judged by SDS gels.

DNase I protection experiments

DNase I protection experiments were performed according to the method of Galas and Schmitz (1978). Buffers and conditions for the footprint experiments are the same as described by Simons *et al.* (1984). The concentration of the inducer isopropyl-β-D-thiogalactoside (IPTG) was 40 mM. For the DNase I protection experiments, 152 bp (*lac* operator) or 174 bp (λ operator) fragments were excised with *Eco*RI and *Spe*I, 3′-labelled with [α^{32}P]dCTP and purified by PAGE. In order to localize the DNase I footprint, G and CT specific degradation was performed according to Maxam and Gilbert (1977) on the respective DNA fragments.

Electrophoretic mobility shift assays

The experimental procedures are as described by Alberti *et al.* (1991), with the following modifications: the standard binding buffer contained 1 mM EDTA and 200 μg poly[d(I-C)] per ml. The IPTG concentration in the control lanes of Figures 4a and 5a was 40 mM. In Figures 4a, 5a and 5b the same 152 bp 3′ end-labelled, gel purified fragments containing ideal *lac* operator or one of its derivates were used as in the footprint experiments. The DNA used in Figure 3b was a 46 bp fragment containing two λ consensus operators which was excised with *Xba*I from pWB9.

[^{14}C]IPTG equilibrium dialysis

Specific activities of wild-type Lac repressor, hybrid Lac [HTH λ cro] repressor and modified hybrid Lac [HTH λ cro] repressor were determined by [^{14}C]IPTG equilibrium dialysis according to Miller (1972).

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