A Study of the CopF Repressor of Plasmid pAMβ1 by Phage Display

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Received 20 October 1999/Accepted 28 February 2000

We studied DNA binding of a transcriptional repressor, CopF, displayed on a filamentous phage. Mutagenesis of a putative helix-turn-helix motif of CopF and of certain bases of the operator abolished the protein-DNA interaction, establishing the elements involved in CopF function and showing that phage display can be used to study repressor proteins.

pAMβ1, pIP501, and pSM19035 are members of a family of low-copy-number plasmids isolated from enterococci and streptococci (for a review, see reference 16). The Rep proteins of pAMβ1 and pIP501 are rate limiting for replication (3, 6, 21). Their synthesis is controlled transcriptionally at two levels. The first level is a transcription attenuation system involving a countertranscript RNA (5, 4, 23). The second involves the 10-kDa products of the copF and copR genes. The two proteins bind to an operator sequence located just upstream of the Rep promoters, Pp in pAMβ1 and PII in pIP501, and repress Rep mRNA synthesis approximately 10-fold (2, 22). CopF shares up to 95% identity with CopR of pIP501 and CopS, the corresponding protein of plasmid pSM19035. The DNA binding motifs of the CopF, CopR, and CopS proteins have not been characterized. However, there appears to be a significant probability that a helix-turn-helix (HTH) motif can form within the region of the CopF protein delimited by residues at positions 16 and 37 (22) (Fig. 1). This region is strictly conserved in CopR and CopS proteins. Sequence alignment of the operator regions of pIP501 and pSM19035 with that of pAMβ1 shows that the three operators are highly conserved (Fig. 1) and that all contain an imperfect inverted repeat of 11 bp. Chemical footprint analysis of a His-tagged CopR protein from pIP501 has shown that it contacts two partially symmetric sites in the major groove of DNA, CGTG12CGTG12 on the top strand and CGTGTC on the bottom strand (the numbers refer to positions within the operator [31]). This protein was shown to interact cooperatively with the operator probe and to bind to DNA as a dimer, with a dissociation constant (KD) of 4 × 10^-10 M (31, 32). Here we present evidence for the involvement of an HTH motif in CopF binding to the operator and report information concerning contacted nucleotides. These results were obtained by phage display of CopF followed by in vitro study of the interaction.

To obtain phages bearing the CopF repressor (designated fusecopF), a BglII PCR fragment containing the copF open reading frame (ORF) was cloned into the polyvalent phage display vector fuse 5 (29). Phage bearing CopF, denoted fusecopF (Fig. 1), was tested for affinity with the minimal-size operator (31-bp, pAMβ1 operator depicted in Fig. 1). Some 52% of the labeled operator was bound to the phage in the absence of the nonlabeled operator (Fig. 2). This amount decreased progressively with increasing amounts of the unlabeled operator fragment but not of a control competitor fragment lacking the operator (a 33-bp DNA fragment corresponding to a part of the plasmid pT181 replication origin [18]). Similar results were obtained with a 238-bp pAMβ1 DNA fragment containing the operator, where 22 to 32% of the fragment was bound to fusecopF phage while 4% of the control fragment lacking the operator was bound. Furthermore, the phage lacking CopF bound <4% of either fragment. This result shows that fusecopF interacts with the minimal-size operator in a sequence-specific manner.

The apparent KD of phage-displayed CopF was determined using the formula KD = [O]1/2 × [R]T, where [O]1/2 is the total operator concentration at which half of the repressor is bound and [R]T is the total repressor concentration (27). In order to estimate the [O]1/2, increasing amounts of DNA were incubated with phages at a fixed concentration (Fig. 3A). [O]1/2 was 6.5 × 10^-10 M. [R]T was estimated as follows. Assuming that each complex contains one phage only, the concentration of active phage should be equal to the maximal concentration of complex formed in the experiment, that is, at the plateau of the curve (Fig. 3A), which corresponds to about 16,000 cpm. A conversion factor between radioactivity and molarity of complex was determined experimentally (Fig. 3B). At the plateau...
of this curve all DNA molecules are saturated with repressor, so the concentration of complexes should be equal to the molarity of the DNA. The deduced concentration of phage active for binding is equal to $6.5 \times 10^{-10}$ M and represents 65% of the phage particles used in the assay. A $K_D$ of $3.25 \times 10^{-10}$ M was determined in the experiment whose results are shown in Fig. 3, and an average value of $3.37 \times 10^{-10}$ M was deduced from two independent experiments, close to that reported for His-tagged CopR ($4 \times 10^{-10}$ M [32]).

We tested whether the putative HTH motif can be isolated
as an independent protein domain. For this purpose, the 54 N-terminal amino acids of CopF, which encompass the motif, were fused to Gp3. The 40 C-terminal amino acids were also fused to Gp3, as a control. Interaction with the 31-bp operator was measured by a filter binding assay with the resulting phages, and results were compared with those for fusecopF. No binding was found with either the amino-end fusion (3% of the fusecopF binding activity) or the carboxyl-end fusion (4.3% of the fusecopF binding activity, with the background representing 3.4%). This result indicates that the interaction requires the whole protein. To investigate the involvement of the putative HTH motif of CopF in protein-DNA interaction directly, a mutagenesis of the motif and the operator was therefore carried out.

Comparisons of crystal or nuclear magnetic resonance structures of complexes between seven different HTH proteins and their DNA binding sites (434R, 434C, LacI, Lambda R, catabolite gene activator protein [CAP], Hin, and Oct-1) have led Suzuki et al. (34) to propose that amino acids at positions 1, 2, 5, and 6 of the DNA-contacting alpha helix are often involved in specific interaction with a set of one to four consecutive bases within a half-operator sequence, while amino acids at positions –1 and 9 of the recognition helix often contact phosphates. Among these proteins (Fig. 4), four possess an arginine at position 6 of their recognition helix, which interacts with a guanine in the operator (LacI [9, 26], CAP [28, 10, 13, 14], Oct-1 [17], and Hin [15]). Furthermore, at least five prokaryotic repressors homologous to the Lac repressor have an arginine at position 6 of their recognition helix, which may be predicted to interact with a G in their operator (24): GalR (37), CytR (36), RafR (1), EbgR (33), and DeoR (35). These nine operators can be aligned with respect to this G (Fig. 4), which is presumably critical for binding, since its change to A, C, or T leads to derepression of P_Lac in vivo while its change to A or C abolishes binding of CAP to its target (25, 28). Additional interactions between recognition helix amino acid 1 and 2 side chains and base positions (Fig. 4) are in great part responsible for specific recognition by each repressor of the cognate operator. Indeed, the specificity of LacI can be changed to that of any of the above-named LacI family repressors by replacement of the corresponding amino acids at positions 1 and 2 of the recognition helix and of the two bases located 3’ to the crucial G (24).

CopF possesses two arginines at positions 1 and 6 of its putative recognition helix, and its operator contains four guanines in its left half (Fig. 4A). Moreover, the CopF operator shares 5 bp of sequence (Fig. 4B) with LacI and the LacI operator, spanning base pairs involved in specificity (Fig. 4A). The alignment of the CopF operator with that of CAP or LacI suggests that guanines 13 or 11 may be involved in binding to Arg6. In order to test the involvement of the putative CopF HTH and operator motifs in binding, substitutions of arginines 1 and 6, serine 5, and guanines 13 and 11 were carried out. An

![FIG. 2. Competition experiments. Increasing amounts of cold competitor were added to the labeled operator target in binding reactions with fusecopF. Reaction mixtures containing Tris-HCl (pH 7.5, 10 mM), NaCl (100 mM), the labeled 31-bp operator (1.2 × 10^{-9} M), and phages (1.6 × 10^{-8} M) were incubated for 10 min at room temperature before filtering of DNA-protein complexes and counting of radioactivity. The percentage of radioactivity bound to the filters is plotted as a function of competitor amount. Squares, 31-bp operator competitor; circles, 34-bp control segment lacking the operator.](http://jb.asm.org)

![FIG. 3. Determination of K_D. (A) Titration curve of phages with DNA. Binding reactions were carried out with increasing amounts of the labeled operator target and a constant phage concentration (10^{-9} M). The operator concentration at which half of the phages are complexed ([O_T]_{1/2}) corresponds to 6.5 × 10^{-10} M. (B) Titration curve of DNA by phages. Binding reactions were carried out with increasing amounts of phages at a constant DNA concentration (5.7 × 10^{-11} M). At the plateau, the concentration of DNA-protein complexes is 5.7 × 10^{-11} M and corresponds to an activity of 1,400 cpm. The concentration of phage active for binding the 31-bp target, [R_T], can be deduced from the curve in panel A. The plateau at which all active phages are complexed to DNA corresponds to an activity of 16,000 cpm, equivalent to 11.4 × 10^{-12} to 5.7 × 10^{-13} M, that is, 6.5 × 10^{-10} M. So K_D deduced from this experiment corresponds to 3.25 × 10^{-10} M.)](http://jb.asm.org)
operator variant, containing a mutation known to affect the copy number of plasmid pAM61 in vivo (a transversion from thymine to guanine at position 10) was also tested.

Two mutant phages were obtained after mutagenesis at position 1, containing Leu or Ile, and three were obtained after mutagenesis at position 6, two having Pro and one having Gin. These phages showed no significant binding to the operator (Table 1). Out of three mutant phages at position 5, two carrying Gin or Pro did not bind the operator, while the third, carrying Thr, could bind only inefficiently. This shows that seven different mutations targeted to the putative recognition helix affect binding of CopF to the wild-type (wt) operator, by affecting either HTH formation (the probability is indicated in Table 1) or the binding specificity.

Since arginines 1 and 6 of the recognition helix appear to be involved in binding, we tested whether the guanines with which they were predicted to interact were also important for binding. Three operator variants were synthesized for this purpose (Fig. 1). In the first (P25/26) G11 was replaced by C. Simultaneously, C21, which is placed symmetrically to G11 relative to the operator center, was replaced by G. In this way, both putative half-operator sites were modified, in order to avoid a possible residual interaction with CopF due to a functional half-site. In the second (P25/26), G13 was replaced by T and the symmetrical T19 was changed to A. The third (P37/38) contained the mutation T10 to G, known to affect the copy number of pAM61 in vivo (30) and a symmetrical change, A22 to C. The binding activities of the three operator variants were compared to that of the wt operator and a control 33-bp fragment lacking the operator (P1/OC1). CopF did not bind operators P23/24 and P37/38 (about 2% of the wt operator, similar to the level of binding of the control fragment). Operator P25/26 was only partially inactivated, since it kept about 30% of the wt operator activity. These data suggest that nucleotides

### Table 1. Effect of amino acid replacement at positions 1, 5, and 6 of the putative recognition helix on the binding activity of CopF to the 31-bp wt operator

<table>
<thead>
<tr>
<th>Amino acid at recognition helix position:</th>
<th>% Residual activity with the mutant/that with the wt&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Probability&lt;sup&gt;b&lt;/sup&gt; of HTH formation</th>
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<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Ile</td>
<td>15.8</td>
<td>25</td>
</tr>
<tr>
<td>Leu</td>
<td>15.3</td>
<td>25</td>
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<tr>
<td>Arg</td>
<td>100</td>
<td>71</td>
</tr>
<tr>
<td>Gln</td>
<td>11.6</td>
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<tr>
<td>Pro</td>
<td>14.9</td>
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<td>Thr</td>
<td>24.1</td>
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<tr>
<td>Ser</td>
<td>100</td>
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<tr>
<td>Gin</td>
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<td>Pro</td>
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<tr>
<td>Arg</td>
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<sup>a</sup> Phages bearing mutant CopF were prepared and tested by a filter binding assay with labeled operator. Residual activity corresponds to the ratio of the radioactivity bound by the phage displaying the mutant relative to that of the phage displaying the wt protein. In this experiment, the background radioactivity in control reaction mixtures free of phages corresponded to 11.5% of the radioactivity found with the phage carrying the wt protein.

<sup>b</sup> The probability of HTH motif formation in mutant CopF protein is given according to the method of Dodd and Egans (11).
amino acid substitutions clustered in the putative HTH motif disrupt the CopF-operator interaction. These substitutions were targeted to arginines at position 1 and 6 of the putative recognition helix and to serine at position 5. These results support the involvement of the HTH motif in binding to DNA. Furthermore, conservation of the binding site seems to be a feature of HTH proteins (38). A DNA recognition box, TNNAN, has been proposed for these proteins (12). This box is contained in the CopF operator (TGTGAA). Taken together, these results lead us to propose that an HTH structure can indeed form on CopF and that it mediates CopF binding to its operator. Substitution of the first two bases of the TGTGA motif abolishes the interaction, while replacement of the second G (position 13 of the operator; see Fig. 1) only weakens the binding. Because Arg in the HTH motifs of several repressors is known to interacts with a G, it is tempting to speculate that in CopF, Arg6 interacts with G11. However, this conclusion should be considered preliminary, since it is supported by negative evidence only and should be confirmed, for instance, by the finding of CopF variants able to interact with a mutant operator containing a G11. Such study could be undertaken by using a phage display approach.

REFERENCES
