Interaction of the Cro repressor with the lysis/lysogeny switch of the Lactobacillus casei temperate bacteriophage A2

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The transcriptional switch region of Lactobacillus casei temperate bacteriophage A2 contains three similar 20 bp operator subsites, O1, O2 and O3, which are interspersed between the divergent promoters Pcl and Pr. The Cro protein binds initially to O2, which overlaps the –35 region of Pr, excluding the RNA polymerase (σA–RNAP) from it. This results in the switching off of cl transcription and directs the incoming phage into the lytic cycle. At higher concentrations, Cro also binds to O1 and/or O2, which overlap Pr, probably introducing a bend in the intervening DNA. This interaction induces DNA looping, which provokes the subsequent displacement of σA–RNAP from Pr. Consequently, Cro abolishes the binding of σA–RNAP to the genetic switch of A2 and, presumably, its own synthesis, contributing indirectly to the entry of phage development into its late stages.

The genetic switch of bacteriophage A2, which infects industrially relevant strains of Lactobacillus casei (Herrero et al., 1994), has a unique operator region (Op). Op is at the centre of a regulatory circuit governing the commitment of the phage between lytic or lysogenic development (Fig. 1A) (Ladero et al., 1998, 1999; García et al., 1999). The A2 Op region comprises two divergently oriented promoters: Pl, which promotes transcription from cI, and Pcl, which directs expression of cro and the replication cassette (Ladero et al., 1998; Moscoso & Suárez, 2000). Interspersed in this segment, three similar, although not identical, 20 bp operator subsites (O1, O2 and O3) have been identified. The CI protein, which confers host immunity against phage A2, binds specifically to Op. At low concentrations, it interacts selectively with O1 and O2 (which overlap Pr). This results in displacement of the RNA polymerase (σA–RNAP) from this promoter and enhances its positioning onto PL (García et al., 1999). At higher concentrations, CI also occupies O3 (which overlaps Pl) with the subsequent exclusion of σA–RNAP from the Pl promoter. It is likely, therefore, that this is the mechanism by which CI represses in vivo the lytic development of phage A2, while promoting and maintaining its lysogenic state.

Cro also binds Op specifically and with an affinity similar to that of CI, although it does not bind co-operatively (Ladero et al., 1999). Cro preferentially binds to the O3 subsite, while at high concentrations it also binds to O1 and/or O2 (Ladero et al., 1999). In this communication, a further characterization of Cro and its role on the lysis/lysogenic decision is described.

The Cro protein of phage A2 was purified as described previously (Ladero et al., 1999). DNA extraction, analysis and purification of DNA fragments were carried out by standard methods (Sambrook et al., 1989). In vitro run-off transcription, electrophoretic mobility shift and DNase I footprint assays were performed as described previously (García et al., 1999).

The Cro protein of lambdoid phages is known to inhibit the transcription of cl by binding to OR, which overlaps Pcl (Plashne, 1986). We hypothesized that the Cro analogue from A2 might play a similar role at Pl, taking into account that Cro binds to the operator region and that it shows a higher affinity for O1 than for O2 and O3 (Ladero et al., 1999). To address this question, we used a DNA fragment containing the switch.

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Fig. 1. (A) Map of the genetic switch of bacteriophage A2. Operator sequences are indicated in the shaded boxes. The −35 and −10 promoter consensus regions are indicated by open boxes. Transcription start sites of the divergently oriented promoters are signalled by open arrows. Numbers within the double-headed arrows indicate the length (in bp) of the relevant features. (B) In vitro transcription assay of the P_L promoter in the presence of Cro. A 390 bp DNA fragment (3 nM) containing the genetic switch region of phage A2 and σ_A-RNAP (60 nM) was used for the in vitro generation of run-off transcripts, which were subjected to reverse transcription in the presence of [α-32P]dATP. Successive lanes show the effect of increasing concentrations of Cro (0, 250, 500, 1000, 1500 and 2000 nM) on cI-specific transcript generation (lanes 1–6).

Having demonstrated that Cro represses transcription from the P_L promoter, we studied its interaction with RNAP through electrophoretic mobility shift assay (EMSA) experiments (Fig. 2). The phage A2 switch region, obtained as an α-32P-labelled DNA segment, was incubated with Cro, σ_A-RNAP or σ_A-RNAP and increasing concentrations of Cro. Protein–DNA complexes were separated by non-denaturing PAGE and analysed by autoradiography. The DNA fragment formed two complexes with Cro: complex I, corresponding to its binding to O_1, and complex II, in which it is bound to O_2 and/or O_3 (Fig. 2, lane 3) (Ladero et al., 1999). Similarly, two σ_A-RNAP–DNA complexes (RPI and RPII) (Fig. 2, lane 2) (García et al., 1999) were observed. The RPI complex is probably made of a single σ_A-RNAP molecule bound to the DNA segment, while RPII would contain two σ_A-RNAP molecules, one bound to each promoter. The addition of one Cro promoter per σ_A-RNAP molecule to the pre-formed σ_A-RNAP–DNA complex resulted in ~45% loss in the intensity of the RPII band and an equivalent increase in the intensity of the RPI band, or, possibly, of a band that migrates slightly slower than RPI (named RPI*, see below) (Fig. 2, lane 4). In the presence of a 2–5-fold excess of Cro per σ_A-RNAP molecule, the amount of σ_A-RNAP bound to both promoters became dramatically reduced (≤5%) (Fig. 2, lane 6), although complete displacement was not reached even at a 10-fold excess of Cro per σ_A-RNAP molecule (Fig. 2, lane 8). Under these conditions, complex II formed by Cro bound to the three operator sequences became increasingly abundant. In parallel, a diffuse fast moving protein–DNA complex (termed II*) that migrated between
both Cro–DNA complexes accumulated. These data can be best explained by assuming that Cro initially displaces $\alpha^A$-RNAP from one of the promoters to originate RPI*, which is postulated to be formed by the binding of one molecule of both $\alpha^A$-RNAP and Cro to the DNA fragment. Furthermore, although the nature of complex II* is unknown, it is proposed that it involves a loose interaction of $\alpha^A$-RNAP with the DNA–Cro complex, because the II* diffuse complex is only formed when $\alpha^A$-RNAP is present in the reaction mixture (an explanation for the fast migration of complex II* in gels is provided below).

To investigate further the type of complexes formed by Cro and $\sigma^A$-RNAP on the Op region, DNase I footprint experiments were performed with a constant amount of $\alpha^A$-RNAP and increasing concentrations of Cro (Fig. 3). At a low Cro concentration (50 nM), the footprint obtained was similar to the one that resulted when $\sigma^A$-RNAP protected both promoters (Fig. 3, compare lanes 1 and 3). In the presence of 100 nM Cro, footprinting of Cro at $O_2$ and $\sigma^A$-RNAP at $P_R$ was observed (Fig. 3, lane 4). This confirmed the assumption on the nature of RPI* from the EMSA experiment (Fig. 2) as being formed by a ternary complex in which one monomer of $\sigma^A$-RNAP would be bound to $P_R$ and one Cro molecule to the $O_3$ subsite.

At higher concentrations of Cro ($\geq 200$ nM), the pattern of $\sigma^A$-RNAP protection is not detected. Under these conditions, the protection by Cro of the $O_1$, $O_2$, and $O_3$ substrates becomes evident, but a new DNase I digestion pattern, which was not detected with Cro alone, is observed (Fig. 3, compare lanes 6–9 with lane 11). Interspersed between the protected stretches, new phosphodiester bonds that are hypersensitive to DNase I cleavage were identified in the $O_3$ subsite and in the space between $O_2$ and $O_3$. These hypersensitive sites are separated by 10±1 or 16±1 nt, which is about one or one-and-half helical turns (assuming 10·5 bp per turn) in double-stranded DNA. DNase I footprinting with similar characteristics has been observed for curved DNA that is wrapped around a protein and in looped DNA (Morrison & Cozzarelli, 1981; Drew & Travers, 1985; Hochschild & Ptashne, 1986). Drew & Travers (1985) explained this phenomenon by proposing that helical grooves located in the inner surface of the bend/loop are sterically occluded and that grooves on the outer face are sites of enhanced DNase I cleavage. DNA bending would then be a consequence of its interaction with both Cro and $\sigma^A$-RNAP (it only happens in the presence of both proteins), which would determine the wrapping of DNA around Cro. The gradual displacement of RNAP, which would be substituted at $P_R$ (or $O_1/O_2$) by a new Cro promoter, would also be determined. This is illustrated by the presence of complex II*, as seen by EMSA, in addition to the expected complex II. The faster migration of II* with respect to II might be a consequence of the decrease in the volume of the complex due to the DNA bending that would remain after RNAP displacement. The width of the II* band would be a consequence of the slow displacement of RNAP by Cro.

From the data reported here and in previous articles, it appears that the general features of the lysis/lysogeny decision of phage A2 resemble those of the $\lambda$ genetic switch (Hochschild et al., 1986; Ptashne, 1986) but its implementation and regulation seems to be simpler (Garcia et al., 1999; Ladero et al., 1999; this work). It appears that the commitment between the two life cycles of phage A2 is taken very early after infection.
Host RNAP presumably binds to both promoters, $P_L$ and $P_R$, in the genetic switch region of the phage genome, since the space between the promoters easily admits two RNAP molecules coexisting in it, as has been shown in vitro (Fig. 2, lane 2) (García et al., 1999). This results in RNA synthesis from both operons; the constitutive transcription of the lysogenic operon, governed by $P_L$, is noticeable (Ladero et al., 1998). This contrasts with the case of phage λ, where CII is needed for the initial synthesis of CI mRNA and where the N protein allows efficient read-through of the terminators placed in the early lytic region.

Presumably, the constitutive synthesis of CI has to be counterbalanced by an efficient production of Cro in order to direct a significant proportion of the infectious events towards the lytic route. This necessity might explain why the transcripts arising from $P_R$ are at least 10 times more abundant than those generated from $P_L$ (García et al., 1999). In this context, it must be noted that the in vitro affinity constants of Cro and CI for the genetic switch of phage A2 are very similar (7 and 6 nM, respectively) (García et al., 1999; Ladero et al., 1999). Thus, the in vivo function of these repressors in determining the commitment of the phage is determined primarily by their ability to distinguish between the $O_1$–$O_2$ and the $O_3$ operator sequences. CI binds preferentially to the $O_1$ and $O_2$ subsites, shutting off transcription from $P_R$, while enhancing expression from $P_L$ so that lysogeny becomes established (García et al., 1999).

Conversely, Cro binds preferentially to the $O_3$ subsite. This results in repression of transcription from $P_L$ (Fig. 1B) and abolition of CI production. However, the data reported here suggest that RNAP remains bound to the $P_R$ promoter, originating a ternary DNA–Cro (at $P_L$)–RNAP (at $P_R$) complex, which accounts for the appearance of RIP* in EMSA (Fig. 2, lane 4). Under these conditions, Cro (and replication proteins) accumulate(s). As a consequence, the phage will enter the lytic cycle. As the concentration of Cro rises, it will progressively interact with the remaining, low affinity, subsites ($O_2$/$O_1$). This would promote displacement of $\sigma^L$-RNAP from $P_R$, which is detected by the generation of the II and II* complexes in EMSA (Fig. 2, lanes 6–8) and repression of cro transcription. The biological meaning of this process might be that the unbinding of $\sigma^L$-RNAP from $P_R$ would indirectly enhance phage maturation by shutting off early gene expression.

In conclusion, it appears that the physiological differences induced by CI and Cro which lead towards the lysogenic versus the lytic cycle may be attributable to (i) the different degree of utilization of the promoters $P_L$ and $P_R$, (ii) the different affinity of the repressors for the operator subsites and (iii) the subsequent displacement of $\sigma^L$-RNAP from its cognate promoters induced by them.

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