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The Type I toxin–antitoxin *par* locus from *Enterococcus faecalis* plasmid pAD1: RNA regulation by both *cis*- and *trans*-acting elements

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Abstract

The pAD1 *par* determinant was the first Type I toxin–antitoxin system identified in Gram-positive bacteria and has recently been shown to be the prototype of a family of loci that is widespread in these organisms. All family members have (i) convergently transcribed toxin message and regulatory RNAs, (ii) three non-contiguous complementary regions for potential interaction, and (iii) intramolecular structures within the toxin message that modulate translation and transcript stability. Therefore, the detailed information available on the *par* locus provides a paradigm for studying the function and mechanism of regulation of the related loci.

Keywords

Toxin; antitoxin system; Post-segregational killing; Antisense RNA; sRNA; Plasmid stability

1. Introduction

The pAD1 *par* determinant was originally identified as a locus required for the stable inheritance of the plasmid's basic replicon (Weaver et al., 1993). Later work identified it as a Type I toxin–antitoxin (TA) system that functioned to stabilize the plasmid by a post-segregational killing (PSK) mechanism (Weaver, 1995; Weaver and Tritle, 1994; Weaver et al., 1996, 1998). However, unlike previously described Type I PSK systems, including the prototypical *hok/sok* system of *Escherichia coli* plasmid R1 (Gerdes and Wagner, 2007), the regulatory RNA of the *par* system, RNA II, was transcribed convergently to the toxin message (Fig. 1), RNA I, overlapping only at a bidirectional intrinsic transcriptional terminator. In addition to the complementary terminator stem loops, complementarity was also provided by direct repeats that were transcribed in opposite directions in the two RNAs (DRs in Fig. 1). Therefore, in contrast to most other antisense regulated systems known at the time, it was predicted that regulation of RNA I translation by RNA II would involve interaction between dispersed regions of complementarity. Detailed structural and interaction experiments later confirmed this prediction (Greenfield and Weaver, 2000; Greenfield et al., 2000, 2001). In addition, intramolecular structures were identified in RNA

I that modulated toxin translation and transcript stability (Greenfield and Weaver, 2000; Shokeen et al., 2008, 2009). The cooperation of all of these sequence elements was found to be essential for proper expression of the PSK function.

In this review, each of the sequence elements will be discussed and their various roles will be described as it relates to *par* function. In addition, recent searches have identified an extended family of *par* homologs located on the plasmids and chromosomes of the Firmicutes; Gram-positive bacteria of low G+C content that include such pathogens as *Staphylococcus* and *Streptococcus* as well as the enterococci (Fozo et al., 2010; Kwong et al., 2010; Weaver et al., 2009). The conservation of the prototypical *par* elements will be examined in the context of the presumably different functions of these loci. Those loci that have been visually and/or experimentally examined and have been shown to possess these conserved elements are shown in Table 1. Since this review focuses specifically on RNA interactions the Fst toxin is not discussed in detail. Information on the toxin may be obtained in recent reviews (Brantl and Jahn, in press; Weaver, 2012).

2. Interaction sites

RNA I and RNA II contain three regions of complementarity, DRa, DRb, and the terminator stem-loop. Detailed *in vitro* structural and kinetic analysis has shown that interaction between the two RNAs is initiated at the terminator loop facilitated by the U-turn motif (Franch et al., 1999) present in RNA I (Fig. 2). Interaction then occurs at the DRa sequence at the opposite end of the RNA II molecule and then is extended into the DRb sequence (Greenfield et al., 2001). If G•U base pairs are considered, complementarity actually extends across the gap between DRa and DRb in RNA II (see Fig. 2). Indeed, formation of an RNA I–RNA II complex protects this entire region from single strand specific cleavage with Pb(II) in RNA II, while in RNA I the three unpaired bases remain accessible (Greenfield et al., 2001).

These results indicated that the terminator loop and DR interacting sequences perform distinct functions; the terminator loop is responsible for setting the interaction rate while the DRs are responsible for stabilizing the complex and inhibiting translation. Further kinetic analysis with specific mutants supported this conclusion (Greenfield and Weaver, 2000; Greenfield et al., 2001). While mutations in the terminator loop resulted in decreased interaction rates, stable complexes were eventually formed *in vitro*. Conversely, mutations in the DRs did not interfere with the formation of unstable kissing complexes but did not progress to the formation of stable complexes. Examination of the same mutants *in vivo* revealed that mutations in the RNA II terminator loop were unable to protect cells from the presence of RNA I, while mutants in either single DR were still capable of protection albeit at a lower level. RNA II mutants with two nucleotide mutations in both DRs could not protect, presumably because they could no longer efficiently interfere with ribosome binding. These results demonstrated that the rate of interaction is more important than the formation of stable complexes. This conclusion was consistent with prior observations in the regulation of R1 replication by the CopA antisense RNA (Wagner et al., 1992).

Comparison of the prototypical pAD1 *par* sequence to plasmid, chromosomal, and phage orthologs identified on the basis of homology to the Fst toxin revealed conservation of the basic structure of the locus in Gram-positive bacteria. Thus, such loci consistently encode convergent promoters producing transcripts that read across a pair of direct repeats and overlap at a bidirectional terminator (Koyanagi and Levesque, 2013; Kwong et al., 2010; Sayed et al., 2012; Weaver et al., 2009). Generally, a region of weaker potential interaction (i.e., isolated base pairs and G•U pairs) is present in the gap between the DRs, though the size of the gap and the strength of the interaction varies. As may be expected, the sequence of the DRs is highly variable, presumably to allow the functioning of multiple *par* homologs in the same cell. Indeed, the *Enterococcus faecalis* chromosome harbors a chromosomal *par* homolog that does not appear to interfere with pAD1 *par* function (K. Weaver, unpublished observation). Curiously, Fst homologs in the Gram-negative enterobacteria (the Ldr toxins) (Fozo et al., 2010) are present in a chromosomally-encoded TA locus organized in a fashion similar to the *hok/sok* system of plasmid R1 (Gerdes and Wagner, 2007) raising interesting evolutionary questions.

While the overall organization of *par* homologs is conserved, the interaction pathway may not be. While the U-turn motif present in the terminator loop of pAD1 RNA I appears to play a key role in initiating the interaction of the two RNAs, this motif is not conserved in either the toxin or antitoxin terminator loop in other *par* homologs (Table 1). The possibility that other RNA structural motifs that increase interaction rate may be present there or elsewhere on these RNAs has not been investigated. RNA–RNA interaction has only been examined in detail in one other *par* homolog, the SprA1/SprA1_{AS} locus of *Staphylococcus aureus* (Sayed et al., 2012). Although not originally attributed as such, this locus contains all of the sequence elements typical of *par* loci and is clearly related. However, the terminator loop of neither the toxin mRNA nor the antitoxin RNA encodes a U-turn motif and the authors present evidence that the terminator stem-loop is not required for interaction between the RNAs. While this may indeed be true, some caveats are worth considering. First, the *in vitro* interaction experiments performed with the SprA1/SprA1_{AS} RNAs examined only formation of stable complexes. Indeed, similar experiments with the pAD1 *par* RNAs also showed that the terminator loop was not required for stable complex formation (Greenfield and Weaver, 2000). It was only upon more detailed examination of interaction rates and the formation of kissing complexes that a key role for the terminator loop was elucidated (Greenfield et al., 2001). Such experiments were not performed with the SprA1/SprA1_{AS} RNAs. Second, the SprA1/SprA1_{AS} investigators showed that substitution of the natural terminator of the SprA1 toxin message with a heterologous terminator did not interfere with translational suppression by SprA1_{AS} provided *in trans in vivo*. However, the effect of the terminator swap and the addition of a tag for toxin detection on SprA1 RNA stability was not determined. In the *par* system, while a mutation in the terminator loop of RNA II prevented translational suppression of full length RNA I *in trans in vivo* (Greenfield and Weaver, 2000), RNA II could protect a terminator deletion of RNA I, perhaps due to the decreased stability of the modified transcript (Greenfield et al., 2000). Therefore, it may be necessary to maintain the relative stabilities of the RNAs to properly evaluate their interactions *in vivo*.

In conclusion, it seems counter-intuitive that the interacting partners of *par*-like systems would not take advantage of the complementarity of the overlapping terminator. But the absence of a U-turn motif in some of these structures and the results with the *S. aureus* SprA1/SprA1_{AS} locus suggest that the importance of this interaction may vary in different members of the family. More detailed examinations of other *par*-like loci will be required to resolve this issue.

3. Intramolecular modulation sites

Two intramolecular structures were identified within the pAD1 *par* RNA I that are important for modulating PSK function (Fig. 2). The first is a 5' stem-loop (5'-SL) structure that interferes with toxin translation. The 5'-SL engages most of the toxin Fst ribosome binding site in double-stranded RNA. Disruption of the 5'-SL substantially increased ribosome binding in toe-print analyses and increased translation of the toxin peptide approximately 300-fold in *in vitro* translation experiments (Greenfield et al., 2000). The 5'-SL also sequesters the first two nucleotides of the DRb sequence, and, indeed, disruption of the 5'-SL increases the rate of RNA–RNA interaction 2.5-fold (Greenfield and Weaver, 2000). RNA II is effective in suppressing translation from either the wild-type RNA or 5'-SL disrupted mutant *in vitro* (Greenfield et al., 2000). Mutations that strengthen the 5'-SL are capable of shutting off Fst translation completely and allow its introduction into host cells in the absence of the antitoxin. Interestingly, however, mutations disrupting the 5'-SL cannot be introduced into host cells even if they are producing RNA II from a multicopy plasmid (Shokeen et al., 2008). This discrepancy between *in vitro* and *in vivo* inhibition of toxin translation probably relates to the pathway of interaction as described below.

The second important intramolecular RNA I structure, the 5' upstream helix (5'-UH), is formed by an interaction between the extreme 5' end of the mRNA and complementary sequences downstream of the toxin coding region (Shokeen et al., 2009). The 5'-UH sequesters the 5' end of RNA I and contributes to its stability *in vivo*. This sequence contributes to the differential stability of the toxin message and antitoxin regulatory RNA, a feature that is critical for TA, and particularly PSK, function. Although RNA I is more stable than RNA II, both RNAs are more stable in complex than they are alone. Thus, basal levels of RNA II and an unstable 5'-UH mutant of RNA I are higher in the presence of the complementary RNA. It is possible that the 3 bp bulge present in RNA I upon complex formation prevents rapid degradation by RNase III.

Sequence comparisons of *par*-family members reveal that the 5'-SL and 5'-UH structures are broadly conserved across the family, indicating their functional importance (Kwong et al., 2010; Weaver et al., 2009). The 5' end of the 5'-UH usually begins with a G and may represent the initiating nucleotide in most cases. The 3' end of the 5'-UH is located at or near the termination codon of the toxin open reading frame. The experimentally determined structure of SprA1 toxin mRNA depicts a large stem-loop at the 5' end that covers both the SD sequence and most of the 5' end of the RNA, leaving two 5' nucleotides accessible (Sayed et al., 2012). This single structure may perform the role of both the 5'-SL and the 5'-UH. However, examination of the sequence downstream of the SprA1 toxin open reading frame reveals an eight nucleotide sequence with perfect complementarity to the 5' end of the

message. Whether interaction of the 5' end with this sequence represents an alternative structure or the reported structure is a metastable structure that refolds as the RNA is transcribed *in vivo* is not resolved.

The structure of SprA1 RNA also revealed the presence of two pseudoknots, the first of which includes an interaction between CA rich and GU rich sequences (Sayed et al., 2012). Interestingly, the potential for the formation of a pseudoknot between a GU rich loop and a CA rich bulge is also apparent in the RNA I sequence (Fig. 2). Perhaps this structure helps to maintain the orientation of the interacting sequences or contributes to the stability of the RNA, though evidence on this point is currently lacking. Pseudoknot structures are not immediately obvious in other *par*-like sequences, but such structures are notoriously difficult to predict.

4. Mechanism of action

The results described above suggest a model for the regulatory interactions of the *par* TA system RNAs. After completion of transcription of both RNAs, interaction is initiated at the terminator loops, stimulated by the U-turn motif present in RNA I. Once tethered via their terminator loops, interaction extends first to the DRa, then to the DRb sequence, thereby suppressing translation. Prior to the interaction of the RNAs, the ribosome binding site of Fst is sequestered by the 5'-SL, preventing premature translation of the toxin, explaining why 5'-SL mutants can be translationally inhibited *in vitro* where complexes are formed prior to ribosome binding but not *in vivo* where ribosome binding would occur prior to the completion of transcription and regulatory RNA binding. The increased stability of the interacting RNAs allows a pool of the silenced complex to accumulate, from which the RNA II antitoxin is only slowly removed. The differential stabilities of the RNAs is at least partially due to the 5'-UH in RNA I. As long as the parent plasmid is retained, antitoxin is continuously replenished and toxin translation is suppressed. But if the plasmid is lost, the antitoxin is eventually degraded and the toxin is translated, killing the cell. It is possible that the 5'-SL is cleaved at some point within this process, thereby both facilitating interaction with RNA II and activating translation, much as processing from the 3' end does for the *hok* message in the *hok/sok* system (Franch et al., 1997). However, in spite of multiple attempts no such processed RNA I has been identified.

It is likely that many of the regulatory features described for pAD1 *par* are altered in related systems to suit the particular purposes for which they evolved. As already mentioned, many family members lack identifiable U-turn motifs in their terminator stem-loops, and in at least one case it has been suggested that this particular structure is entirely dispensable (Sayed et al., 2012). While the pAD1 *par* RNAs and the RNAs from the *fstSm/srSm* locus of *Streptococcus mutans* are constitutively produced (Koyanagi and Levesque, 2013), the antitoxin SprA1_{AS} and the RNA II component of the *E. faecalis* chromosomally-encoded *par*_{EF0409} have been shown to be responsive to environmental conditions (Michaux et al., 2014; Sayed et al., 2012; K. Weaver, our unpublished observations). Furthermore, while the formation of a pool of stable complex is essential for the PSK function of pAD1 *par*, there is no reason to believe that this feature would be conserved in chromosomally-encoded family members which presumably perform a different function. Indeed, preliminary results from

our laboratory indicate that overproduction of RNA I_{EF0409} from the *par*_{EF0409} locus reduces the levels of RNA II_{EF0409} as would be expected if the complex was targeted for degradation. Examining the variability in regulatory mechanisms may help to elucidate the functions of the cryptic chromosomal *par* homologs.

More recently, a number of Type I TA systems with toxins unrelated to those encoded in the *par* family have been identified that encode converging toxin message and regulatory RNA transcripts (Durand et al., 2012). Like the *par* systems, they overlap in their 3' ends and the resulting complementarity provides the primary point of interaction of the toxin and antitoxin RNAs. Unlike the *par* systems they do not contain complementary sequences in the 5' end that regulate ribosome access to the toxin ribosome binding site upon complex formation. Rather, regulation is by targeted degradation of the complex either by RNase III or by 3'-5' exoribonucleases. All these systems also encode intramolecular structures that occlude the ribosome binding sites for the toxin and/or other mechanisms to decrease translation efficiency, suggesting that this may be a universal feature of RNA TA pairs whose primary interaction site is at the 3' end (Brantl and Jahn, in press; Durand et al., 2012).

5. Unresolved questions

While the mechanism of interaction of the pAD1 *par* RNAs and the regulation of the Fst toxin has been investigated in detail and the basic characteristics of a few homologs have been defined, a number of questions remain outstanding.

What is the mechanism of antitoxin RNA degradation alone and in complex? Clearly some mechanism preferentially removes pAD1 RNA II and other unstable RNA II-like antitoxins from the RNA I–RNA II complex. How this occurs has been difficult to study, partly because RNA decay pathways are so poorly understood in the relevant Gram-positive bacteria. In several TA systems, as well as other antisense RNA regulated systems, RNase III is involved in degrading the RNA–RNA duplex (Brantl and Jahn, in press), but this destroys both target and regulatory RNAs and would be unsuitable for the *par* system which requires maintenance of the toxin message. Indeed, our work indicates that RNase III is not involved (K. Weaver, unpublished observations). In Gram negative bacteria, Hfq has been shown to recruit RNase E to RNA–RNA complexes (Aiba, 2007), but enterococci and streptococci lack both Hfq and RNase E homologs. RNase Y, RNase J1, RNase R and PNPase have been implicated in antitoxin degradation in Gram-positive organisms (Brantl and Jahn, in press), but it is unclear whether they are involved in the removal of the antitoxin from the complex or in degradation of free antitoxin RNA or both. Thus, what chaperones, helicases or RNases might be involved in removing RNA II from the *par* RNA complex remains unclear.

What, if any, role does transcriptional interference play in regulation? Clearly converging RNA polymerase molecules cannot transcribe the transcription terminators at the same time and mechanisms of transcriptional interference have been described in many systems with converging transcription (Georg and Hess, 2011). In addition, while pAD1 *par* has symmetrical runs of uridine residues on each side of the terminator stem loop, the uridines

on the 3' end of the toxin message are lacking in many homologs suggesting variability in termination efficiency. Toxin suppression has been documented *in trans* on heterologous replicons in all of the systems tested, but the question remains whether transcriptional interference plays a role in the natural context at a normal copy number.

What are the three-dimensional structures of the RNAs alone and in complex? While we have good secondary structures in two systems and have definitively identified the interacting sequences, a full understanding of the interaction pathway will require 3D structures. Such structures may aid in defining possible processing points that destabilize RNA II and/or identify idiosyncrasies or regulatory aspects in individual systems.

What alterations have been made in RNA and promoter sequences that account for the differences in regulation observed and how do these serve the overall evolved function of each system? While many of the chromosomal *par* homologs may be parts of plasmid remnants it is clear that at least some of them are not (Weaver et al., 2009). The function of these systems is unknown. Do they play a role in persistence, as has been observed in the TisB/IstR Type I TA system and in several Type II TA systems (Gerdes and Maisonneuve, 2012; Lewis, 2010)? If not, what role do they play and how do their regulatory characteristics suit their role?

TA systems provide a unique opportunity to examine the evolution of genetic elements in biological systems. They are small, self-contained and many of their plasmid-encoded members have been well-studied. The chromosomal representatives have clearly evolved to perform a distinct function from their plasmid counterparts and have different regulatory schemes to suit those functions. While these functions are not currently known, the study of regulatory phenomena may lead to clues for discerning their role.

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Fig. 1.

Organization of the pAD1 *par* locus and the Fst toxin. Converging promoters (black arrowheads labeled P) transcribe the toxin-encoding RNA I (shaded arrow below line) and the antitoxin RNA II (dark arrow above line) toward a bi-directional intrinsic transcriptional terminator (converging lightly shaded arrows). The RNAs are transcribed across direct repeats DRa (shaded arrows) and DRb (black arrows) at which interaction occurs, suppressing translation of the Fst coding sequence (black box on DNA and RNA).

Fig. 2.

Secondary structures of pAD1 *par* RNA I and RNA II. The specific regions of interaction between the RNAs are shaded to coordinate with Fig. 1 and labeled accordingly. Interaction is initiated at the U-turn motif (labeled YUNR) present in the loop of the terminator of RNA I (lightly shaded). This interaction is indicated by the arrow labeled A. The interaction then extends to the direct repeat sequences DRa (medium shading) and DRb (dark shading). These interactions are indicated by arrows labeled B and C, respectively, and are responsible for preventing translation of Fst, since the initiation codon (I.C.) and the ribosome binding site (RBS) are sequestered by the interacting RNAs. The two boxed structures, 5'-SL and 5'-UH are responsible for preventing premature translation of Fst and RNA I stability, respectively. A double-headed arrow depicts a putative interaction between a G, U rich loop and an A, C rich bulge that could form a pseudoknot analogous to that detected in the SprA1–SprA1_{AS} interaction (Sayed et al., 2012).

Table 1

Currently defined *par* homologs.^a

Organism	Genetic element	Accession number	Location (toxin gene locus)	Flanking genes	U-turn motif ^b	References
<i>E. faecalis</i>	pAD1	L01794	4017–4420 (Fst)	Replication and UV resistance genes	Yes (RNA I)	Weaver et al. (2009)
<i>E. faecalis</i>	pAMS1	EU047916	2099–2406 (Orf5)	Bacteriocin genes and unknown orfs	Yes (RNA II)	Weaver et al. (2009)
<i>E. faecalis</i>	pTEF2	NC_004671	55422–55721 (unannotated)	ParA and UvrC	No	Weaver et al. (2009)
<i>E. faecalis</i>	chr	NC_004668	381900–381605 (EF0409)	PTS components	No	Weaver et al. (2009)
<i>L. gasseri</i>	φgaY	AB177605	2489–2153 (Orf35)	Lysin and att site	Yes (RNA I)	Weaver et al. (2009)
<i>S. saprophyticus</i>	chr	NC_007350	897263–896954 (SSP0870)	6-phosphogluconolactonase and aldehyde dehydrogenase	Yes (RNA I)	Weaver et al. (2009)
<i>L. casei</i>	chr	NC_008526	2668998–2669318 (LSEI2682)	Mannose-6-P-isomerase and two-component signal transduction system	Yes (RNA I)	Weaver et al. (2009)
<i>C. divergens</i>	pCD3.4	DQ087597	773–462 (unannotated)	Bacteriocin and replication region	Yes (RNA I)	Kwong et al. (2010)
<i>M. caseolyticus</i>	pMCCL2	AF009486	5144–4849 (unannotated)	Hypothetical proteins	Yes (RNA I)	Kwong et al. (2010)
<i>S. pneumoniae</i>	chr	NC_003028	231543–231264 (SP0258)	Group II intron maturase and RuvB	Yes (RNA I)	Kwong et al. (2010)
<i>S. pneumoniae</i>	chr	NC_003028	2086045–2086417 (Fst-B)	Fuculose operon and Zn ABC transporter	No	Fozo et al. (2010)
<i>L. lactis</i>	pSK11L	DQ149244	28445–28774 (unannotated)	Lactose PTS repressor and resolvase	Yes (RNA I)	Kwong et al. (2010)
<i>S. aureus</i>	chr	NC_002952	751287–751593 (SAR0716)	Potential integrated plasmid	No	Kwong et al. (2010)
<i>S. aureus</i>	chr	AP009351	1889597–1889770 (SprA1)	υΣαβ pathogenicity island	No	Sayed et al. (2012)
<i>L. brevis</i>	chr	CP000416	2090769–2090451 (unannotated)	Hypothetical proteins and tRNA-Arg	No	Kwong et al. (2010)
<i>L. monocytogenes</i>	chr	CP001175	2190659–2190976 (unannotated)	LysR regulator, acetyl transferase and glycosyl transferase and fructose PTS	Yes (RNA I)	Kwong et al. (2010)
<i>S. mutans</i>	chr	NC_004350	211452–211769 <i>Fst-Sm</i>	Hypothetical protein and a type II T _A system	No	Koyanagi and Levesque (2013)

^aThe loci shown have been examined visually and shown to have all of the sequence elements typical of *par* including converging promoter, shared bidirectional terminator, DRa and DRb repeats, 5'-UH and 5'-SL. Production of the relevant RNAs has been demonstrated for the pAD1 *par*, pAMS1 *par*, EF0409, Fst-B, SprA1, and Fst-Sm loci. Structural information is only available for pAD1 *par* and SprA1 loci. Numerous other potential *par* homologs have been identified by Kwong et al. (2010) and Foza et al. (2010) but have not been investigated for the appropriate sequence elements.

^b Presence of a U-turn motif is inferred from the presence of the 5'-YUNR-3' signature sequence in the loop of the terminator stem-loop. Importance of this sequence has been demonstrated only for pAD1 *par*.