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Genetics

# KorA protein of promiscuous plasmid RK2 controls a transcriptional switch between divergent operons for plasmid replication and conjugative transfer

(divergent transcription/protein–DNA interactions/plasmid propagation/KorA repressor protein)

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**ABSTRACT** **The *trfA* and *trb* operons, encoding genes essential for replication and conjugative transfer of broad host range plasmid RK2, are transcribed divergently. Deletion analysis presented here indicates that *trfAp* and *trbAp* are arranged as face to face promoters. The presence of the *korA* gene, whose product is known to repress seven operons on RK2, including the *trfA* operon, is shown here to stimulate *trbAp*. The effect of *korA* on *trbAp* is mimicked by the *trfAp-1* promoter down mutation, suggesting that a reduction in the activity of *trfAp* is required for derepression of *trbAp* activity. The *trfAp-1* mutation reduces RNA polymerase binding and open complex formation at *trfAp* but does not stimulate melting at *trbAp in vitro*. Therefore, the inhibition of *trbAp* is most probably due to forward transcription initiated at *trfAp*. The simultaneous inhibition/stimulation by KorA is seen even in the presence of the other repressors KorB and TrbA, which act at this region, thus providing a dominant mode of coordinating plasmid replication and transfer. This may be one of the keys to understanding how the maintenance and spread of promiscuous plasmids are balanced in different environments.**

The IncP plasmids are of particular interest because they are capable of conjugative transfer between and maintenance in almost all Gram-negative bacterial species (1). RK2, which is virtually identical to RP1, RP4, R18, and R68, the best characterized IncP$\alpha$ plasmids (1), provides an interesting system to study factors influencing the balance of plasmid spread and maintenance because expression of the adjacent *trfA* (2, 3) and *trb* operons (4, 5) encoding genes for replication and transfer is coordinated (6–8) (Fig. 1).

The *trfA* operon encodes a single-stranded DNA binding protein Ssb (13, 14) and TrfA, which is essential to activate the replication origin *oriV* (15–17). *trfAp* is repressed by KorA (18), KorB (18), KorF (formerly KorFI), and KorG (formerly KorFII) (12), which are encoded in the central control operon (9–12), and TrbA (7), the product of the first cistron in the *trb* operon. KorA and KorB are repressor proteins with well defined operators (19–22), while KorF and KorG are putative histone-like proteins whose specific targets are not known (12). The TrbA binding site overlaps the *trfAp* −10 region but has not been fully defined (unpublished data).

The *trb* operon consists of 15 cistrons, which are involved in mating pair formation during conjugative transfer (5, 23). Promoters precede *trbA* and *trbB* (5, 8, 18) (Fig. 1). The *trbB* promoter is repressed by KorB (8, 18) and TrbA (7). Expression of *trbA* should shut off *trfAp* and *trbBp* but transcription from *trbAp* is also likely to continue into *trbB* and beyond. This transcript is predicted to contain secondary structure, which may determine stability and translational characteristics different from those of the *trbBp*-generated message (6). Thus, *trbAp* activity should be crucial in the balance of expression of these operons for alternative modes of plasmid propagation.

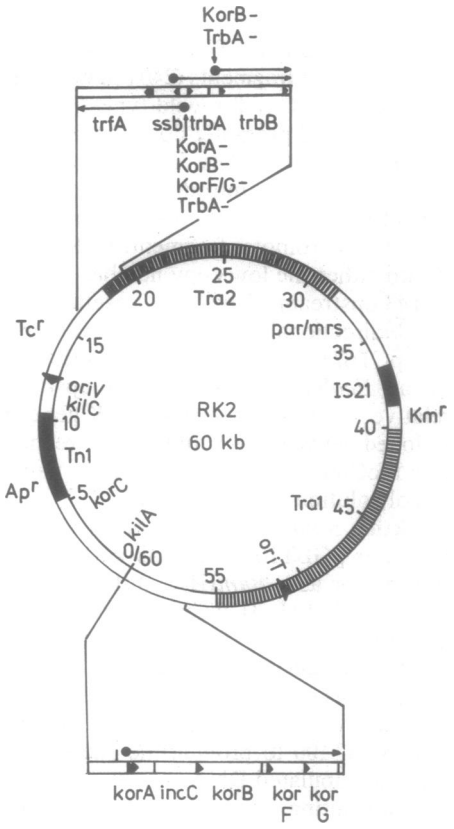


FIG. 1. Map of RK2 showing organization of the *trfA* operon (2, 3), the *trb* operon (4, 5), and the central control operon (9–12). Repressor proteins, which act on *trfAp* and *trbBp*, are shown above or below the relevant promoter. ■, Transposable elements; ◫, transfer regions; ●, promoters; ı, terminators; ▭, open reading frames; ◗, origins for vegetative (oriV) and transfer (oriT) replication.

Here we show that *trfAp* and *trbAp* are face to face divergent promoters and that KorA, one of the key regulatory proteins that represses *trfAp*, stimulates *trbAp*, thus controlling a vital switch in the circuitry that regulates gene expression on this plasmid.

## MATERIALS AND METHODS

**Bacterial Strains, Plasmids, and Growth Conditions.** *Escherichia coli* K-12 strain C600K (*thr-1, leu-6, thi-1, lacY1, supE44, tonA21, galK*) (24) was used. Previously described

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Abbreviations: RNAP, RNA polymerase; tsp, transcription start point(s).

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plasmids are referenced in the text. New plasmids are described when first mentioned. Media used were L broth (25), L agar, and McConkey agar with 1% galactose. Antibiotic resistance was selected as described (7).

**Manipulation and Analysis of DNA and RNA.** DNA preparation, manipulations, and sequencing were by standard techniques (26–28). RNA was isolated (29) from 50-ml L broth cultures of logarithmically growing cells induced for 3 hr with 1 mM isopropyl β-D-thiogalactopyranoside if required. RNA (25–50 μg) was annealed to 5′ $^{32}$P-labeled oligonucleotide, and primer extension (30) was performed with avian myeloblastosis virus reverse transcriptase.

**Assay of Catechol 2,3-Oxygenase Activity.** *xylE* expression was determined by enzymatic assays (31) with logarithmically growing bacteria. Protein was determined by the biuret method (32).

**Cloning by PCR.** Standard PCRs (33) were performed as described (7). Promoter fragments (240 bp) were amplified on pMMV115.1 template (34) for wild-type *trfAp/trbAp* and pVI107.1 (2) for *trfAp-1* promoter down mutation with a pair of primers 1 and 2 (Fig. 2*A*) with *Bam*HI sites at their ends. Primer 5 used with primer 1 introduced a T-to-C change into the putative −10 sequence of the *trbA* promoter. Primers 1 and 6 gave a 290-bp promoter fragment. PCR products were introduced into either the low copy number promoter probe vector pGBT43 upstream of the *xylE* cassette (7) or the high copy number bidirectional promoter probe vector pGBT100. pGBT100 is pCT673 (36) with a promoterless *xylE* gene inserted on an *Eco*RI/*Bam*HI fragment upstream of and inverted relative to the promoterless *galK* gene. Divergent promoters cloned between *galK* and *xylE* were monitored on the indicator McConkey agar (GalK activity) sprayed with 0.1 M catechol solution (XylE activity).

**BAL-31 Deletion Analysis.** The 240-bp *trfAp/trbAp* region was inserted into pGBT100 either as a *Bam*HI fragment, giving pGBT159, or as a *Bam*HI/*Hin*dIII fragment, giving pGBT259. Standard BAL-31 digestion on pGBT159 linearized with the *Hin*dIII or pGBT259 linearized with the *Bam*HI site gave deletions originating from the *ssb* or the *trbA* end of the *trfAp/trbAp* insert, respectively. Deletion endpoints were established by DNA sequencing and the inserts were recloned into pGBT100 to provide the same genetic background for the manipulated DNA.

**DNase I Footprinting.** Fragments were 5′-labeled with [γ-$^{32}$P]ATP. Footprinting was performed as described (37) with two modifications. The binding buffer was as follows: 50 mM Tris·HCl, pH 8/10 mM $MgCl_2$/50 mM NaCl/0.2 mg of bovine serum albumin per ml, and DNase I cleavage products were directly precipitated with ethanol, washed with 70% ethanol, vacuum dried, resuspended in loading buffer, and analyzed on denaturing 6% (wt/vol) polyacrylamide gels.

**Permanganate Footprinting.** The 5′-labeled fragments were incubated for 30 min at 37°C with RNA polymerase (RNAP) and then treated with potassium permanganate (38) with the modifications described (39).

**RNAP Gel-Shift Assay.** Radioactive fragments were incubated at 25°C in standard transcription buffer (30 mM Tris·HCl, pH 8.2/100 mM KCl/3 mM $MgCl_2$/0.1 mM EDTA/10 mM dithiothreitol/45 μg of bovine serum albumin per ml). Heparin was added to a final concentration of 50 μg/ml for 1 min. Glycerol was added and samples were loaded onto a 1% agarose gel and run at 6 V/cm in 1× TAE buffer (40). With DNase I-treated complexes (41), the RNAP binding reaction and heparin addition were conducted as described above. The $MgCl_2$ concentration was then adjusted to 10 mM and DNase I was added (1 μg/ml) for 1 min at 25°C. The reaction was terminated by adding EDTA to 25 mM.

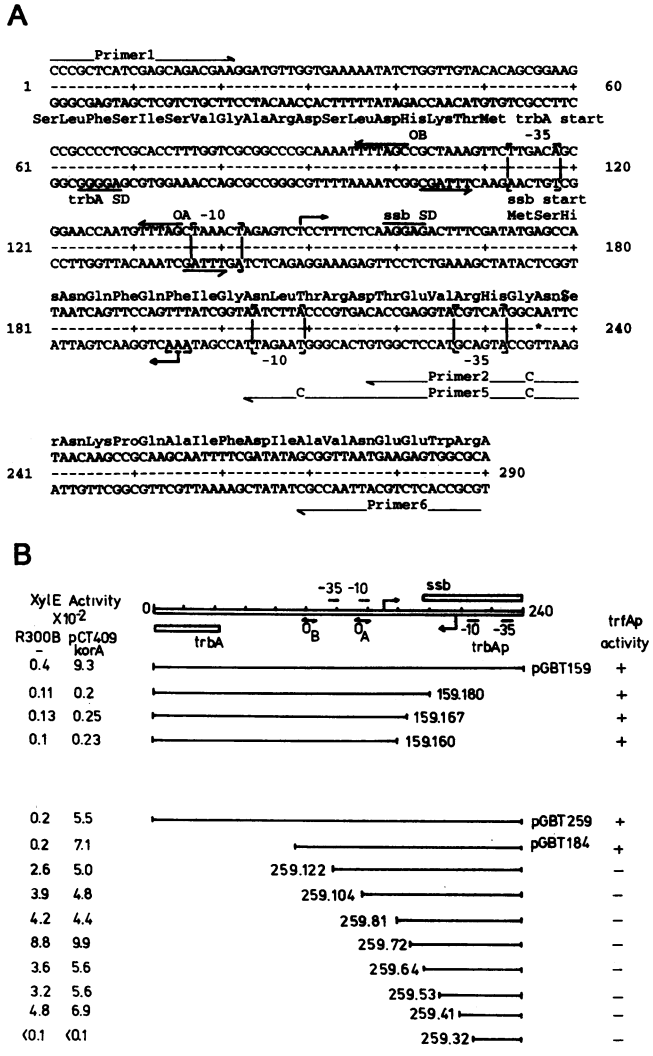


FIG. 2. Deletion mapping of *trbAp*. (*A*) Sequence of the *trfAp/trbAp* region showing −35 and −10 boxes for *trfA* and *trbA* as well as tsp, amino acid sequence of N-terminal parts of TrbA and Ssb, Shine–Dalgarno (SD) sequences, KorB operator (OB) and KorA operator (OA), primers used for PCR. (*B*) Deletion analysis of *trfAp/trbAp* region. Assays on *trbAp–xylE* fusions were done with pCT409 (18) to provide *korA* or with R300B (35) as a negative control. *xylE* activities are those from a single experiment but these relative values were reproducible in all experiments. *trfAp* activity was scored by *galK* expression on McConkey agar plates. Plasmid pGBT184 carries the PCR insert cloned with primer 2 and primer 4 corresponding to the sequence from coordinates 94–115 as presented in *A*.

## RESULTS

**KorA Represses *trfAp* and Stimulates *trbAp in Vivo*.** We previously observed divergent transcription from the *trfAp* region (18), corresponding to *trfA* and *trb* operons (4–7) (Fig. 1), using DNA containing the *trfAp-1* promoter down mutation (a T-to-C transition at −7 of *trfAp*) (18, 19). To study the wild-type sequence we used PCR to generate a 240-bp *trfAp* fragment (Fig. 2*A*, coordinates 1–240), running from −148 to +92 relative to the *trfAp tsp* (42). The fragment was inserted in both orientations into the *xylE* promoter probe vector pGBT43 (7), based on the pSC101 replicon, whose copy number is similar to that of RK2. This created plasmids pGBT58 (measuring *trfAp* activity) (7) and pGBT59 (measuring *trbAp* activity). Constitutive *trbAp* activity was <0.5% relative to *trfAp*. With *korA* present on either a high [pGBT37 (7); Table 1] or a low copy number plasmid [pCT409 (18); data

Table 1. Effect of *korA in vivo* on transcription from *trfAp* and *trbAp* in wild-type and mutant regions

| Promoter probe fusion | Plasmid in trans | | | |
|---|---|---|---|---|
| | pGBT30 | pGBT37 | | |
| | + IPTG | − IPTG | + IPTG | 37/30* |
| pGBT58 *trfAp–xylE* | 3.122 | 0.441 | 0.047 | 0.015 |
| pGBT59 *trbAp–xylE* | 0.004 | 0.069 | 0.105 | 26 |
| pGBT70 *trfAp*†*–xylE* | 0.291 | 0.170 | 0.026 | 0.09 |
| pGBT71 *trbAp*†*–xylE* | 0.041 | 0.043 | 0.039 | 0.95 |

*xylE* expression was determined by assay of catechol 2,3-oxygenase activity. IPTG, isopropyl β-D-thiogalactopyranoside.
*Ratio of *xylE* activity in the presence of induced pGBT37 (*tacp–korA*) (7) and pGBT30 (*tacp*) (12).
†The 240-bp fragment carries the *trfAp-1* mutation. pGBT71 is similar to pGBT70 (7) with the 240-bp fragment in the opposite orientation.

not shown] we confirmed that *korA* represses *trfAp* 40- to 60-fold and discovered that it stimulates *trbAp* 20- to 25-fold. With *korA* present *trfAp* and *trbAp* had similar strength.

**A *trfAp* Promoter Down Mutation Removes the Need for *korA*.** Comparison of wild-type and *trfAp-1* mutant promoter regions in the presence and absence of KorA demonstrated that the *trfAp-1* mutation decreases *trfAp* strength ≈10-fold (pGBT70) (7) and increases *trbAp* activity ≈10-fold (pGBT71) (Table 1). As observed for DNA with the *trfAp-1* mutation (18, 19), KorA represses *trfAp* (pGBT70/pGBT37) but does not stimulate *trbAp* activity (pGBT71/pGBT37). This suggests that the product of *korA* is not needed directly to stimulate *trbAp* activity but that it acts indirectly by inhibiting *trfAp* activity, which interferes with *trbAp* activity.

***trbAp* Is Located Downstream of *trfAp*.** To map *trbAp*, we generated deletions from position 1 (*trbA* gene end) or position 240 (*ssb* gene end) and tested them for constitutive as well as *korA*-dependent *trbAp* activity (Fig. 2*B*). Starting at position 240, removal of 60 bp inactivated *trbAp* (pGBT159.180). With deletions originating at position 1, loss of *trfAp* activity coincided with an increase in *korA*-independent *trbAp* activity (pGBT259.122 to pGBT259.41). This *korA*-independent *trbAp* activity was still present when DNA corresponding to nt 199–240 (Fig. 2*A*) remained (pGBT259.41) but was lost on deletion of a further 9 bp (pGBT259.32). We concluded that (*i*) *trbAp* activity lies within a region of 41 bp in *ssb*, downstream of *trfAp*; (*ii*) an active *trfAp* has an inhibitory effect on *trbAp*; and (*iii*) when *trfAp* is inactivated there is no further stimulation of *trbAp* by KorA.

To locate the transcription start point (tsp) of *trbAp* 5′-end-labeled primer 1 (Fig. 2*A*) was hybridized to mRNA isolated from bacteria containing plasmid pGBT59 (*trfAp/trbAp*) and either pGBT30 (vector control) or pGBT37 (*tacp-korA*). KorA stimulated the appearance of three groups of extended species corresponding to end points in the nt 1–240 region (data not shown). The major species corresponded to a tsp at one of the three A residues at positions 194–196 in Fig. 2*A*, placing *trbAp* between nt 200 and 240 as proposed above (Fig. 2*B*), where >95% of *trbAp* activity disappeared when this region was deleted. For this tsp, the most likely −10 box for *trbAp* is located between bases 204 and 209 (Fig. 2*A*), so we introduced a transition from T to C at position 209 using primer 5. The first PCR clone analyzed (pGBT101) had also lost the A at position 205. The mutant DNA fragment (*trbAp-1*) exhibited no *trbAp* activity (<0.001 unit of XylE) even in the presence of *korA*. This strongly supports the proposed location of *trbAp* and confirms it as the sole cause of transcription in this region.

The *trbA* promoter sequences identified in this way are very close to the end of the cloned 240-bp PCR fragment and may lack specific upstream sequences. Therefore, we repeated the PCR cloning with primers 1 and 6, adding a further 50 bp upstream of *trbAp* (Fig. 2*A*). *trbAp* activity of the 290-bp insert (pGBT197; 0.017 unit) was only 2-fold higher than in the 240-bp insert of pGBT59 recloned into pGBT100 (pGBT159; 0.008 unit). The stimulatory effect of *korA* on *trbAp* expression in pGBT197 is the same as in pGBT159 (20- to 25-fold).

**RNAP Footprints at the Divergent Promoters.** DNase I footprinting with wild-type DNA labeled on the bottom strand (Fig. 2*A*; data not shown) showed for *trfAp* a clear window of protection from −55 to +26 with enhancements at −24/−25. The *trbAp* region is also well resolved and showed clear protection from −45 to +7 relative to the *trbAp* tsp (first of three A residues). Footprinting on the top strand was relevant only to *trfAp* because of the distance of *trbAp* from the labeling point. It showed protection from −52 to +40, interspersed with enhancements at positions −26/−24 and +23/+24 (data not shown).

The *trfAp-1* mutation changes the RNAP footprint at *trfAp* (data not shown). The enhancement at −24/−25 is evident only at higher RNAP concentration. The area of protection is shorter, extending from −55 to −5, consistent with a reduced isomerization from closed to open complex, which is normally accompanied by an extension of protection past the +1 position. The footprint on *trbAp* is unchanged from wild type.

**Open Complex at *trfAp* Does Not Exclude RNAP from *trbAp*.** Permanganate, which specifically attacks T residues in single-stranded DNA, was used to detect open complex formation at the divergent promoters. RNAP melts the 290-bp fragment in two locations (Fig. 3*A*). With high RNAP concentration, the top strand shows opening of T residues at positions 142, 147 (the strongest), and 149 for *trfAp* as well as 195/196, 198, 202, 205, and 207 (the last two the strongest) for *trbAp* (lanes 1 and 2). At low RNAP concentration (5.9 nM) the melting was detected only at *trfAp* (lane 3). Fig. 3*B* compares interaction of RNAP with wild-type (lanes 4–6) and mutant (lanes 1–3) DNA sequences. The presence of the *trfAp-1* mutation reduces melting at *trfAp* but does not enhance the melting at *trbAp*.

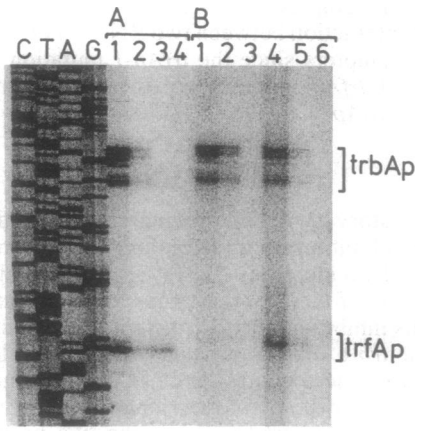


FIG. 3. Permanganate footprinting on the *trfAp/trbAp* region. (*A*) *Bam*HI linearized pGBT259 was cut with *Sal* I after 5′-end-labeling and then incubated (final concentration, 0.7 nM) for 30 min at 37°C with different concentrations of RNAP. Lanes 1, 590 nM RNAP; 2, 59 nM RNAP; 3, 5.9 nM RNAP; 4, no RNAP. Sequencing reactions with primer 1 correspond in size to fragments 3 bp longer than *Bam*HI-cut template. (*B*) PCR fragment (290 bp) with *trfAp-1* mutation (final concentration, 6 nM), labeled on the top strand, was incubated with 590 nM, 59 nM, and no RNAP (lanes 1–3, respectively). Lanes 4–6 correspond to the 290-bp wild-type PCR fragment labeled on the top strand (final concentration, 6 nM) incubated with 590 nM, 59 nM, and no RNAP, respectively. Sequencing reactions correspond directly to the template used.

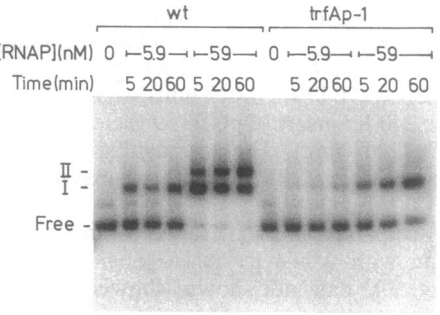


FIG. 4. Open complex formation on promoter fragment *trfAp/trbAp*. Wild-type and *trfAp-1* mutant 290-bp PCR fragments (6 nM) were incubated with RNAP at 5.9 nM or 59 nM for different periods of time at 25°C before adding heparin. Gel image shows free DNA and heparin-resistant complexes with one RNAP molecule (complex I) and two RNAP molecules (complex II).

A gel-shift assay was used to determine whether RNAP can simultaneously form two open complexes on DNA of the *trfAp/trbAp* region. With either increasing time or RNAP concentration, wild-type DNA showed first one and then two retarded species corresponding to binding of one (complex I) and two (complex II) molecules of RNAP (Fig. 4),, indicating that a single DNA fragment can accommodate two open complexes. DNA carrying the *trfAp-1* mutation formed only complex I, and this required the high RNAP concentration, which promotes the transition to complex II on the wild-type DNA. This suggested that complex I is different for wild-type and mutant DNA. To locate RNAP in these open complexes we digested them with DNase I before separation in agarose gels, isolated DNA of the different species, and resolved them by urea/PAGE (Fig. 5). For wild-type DNA, complex I (Fig. 5*B*, lane 2) shows clear protection of *trfAp* against DNase I, while complex II shows protection of both promoters (lane 3). For mutant DNA, complex I (lane 5) shows no protection in the region of *trfAp* but strong protection in the region of *trbAp*. Combining these results with the permanganate footprinting (Fig. 3) we concluded that RNAP initially forms an open complex at *trfAp* followed by transition from closed to open complex at *trbAp*. However, there seems to be no direct interaction between two RNAP molecules bound at the two promoters since the *trfAp-1* mutation, inhibiting the melting at *trfAp*, appears not to affect open complex formation at *trbAp*.

## DISCUSSION

All the regulatory switches previously identified for RK2 involve the inhibition of transcription as concentration of regulatory protein increases (12, 18, 37, 43). Our observation that the activity of KorA at the *trfAp/trbAp* region results in simultaneous inhibition of *trfA* transcription and stimulation of divergent *trbA* transcription reveals a completely new response within these control circuits. Here we have established a number of points concerning this response. First, *trfAp* dominates over *trbAp*. When the two promoters are separated and their activities are compared, *in vivo trfAp* is 50-fold stronger than *trbAp*. When present together, *trfAp* activity is maintained at a high level while *trbAp* activity is inhibited 20-fold. Reduction of *trfAp* activity by KorA repression or mutation results in relief from this inhibition. Thus, modulation of *trfAp* activity provides a means of controlling *trbAp* activity. Second, the *in vitro* data suggest that the effect of *trfAp* on *trbAp* is not due to exclusion of RNAP or inhibition of open complex formation. While protection of *trfAp* by RNAP is at least 10-fold stronger than at *trbAp*, a reduction in RNAP binding at *trfAp* due to the *trfAp-1* mutation does not increase binding at *trbAp* as would

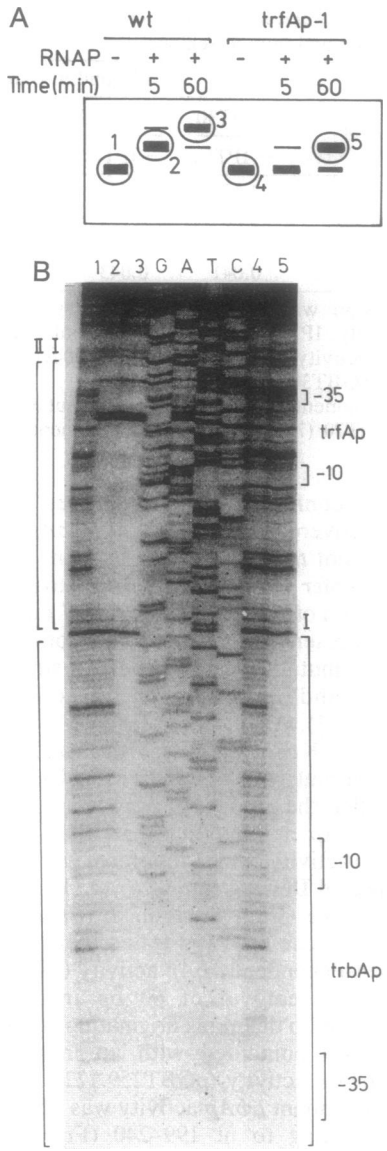


FIG. 5. Footprinting analysis of open complexes. (*A*) Schematic representation of the agarose gel from which DNase I-treated open complexes were isolated with one (complex I) or two (complex II) RNAP molecules bound. (*B*) Identification of open complexes. Lanes 1–5 correspond to DNA fragments isolated by GlassMax (GIBCO/BRL) from the gel as presented in *A* and run on a 6% denaturing polyacrylamide gel. The −10 and −35 boxes for both promoters are indicated as well as the protected regions in complexes I and II. Sequencing reactions were done on pGBT197 DNA with primer 6; the sequence was then shifted up 9 bases in relation to *Hin*dIII-labeled fragments.

be expected if RNAP bound at *trfAp* blocked access to *trbAp*. Similarly, open complexes can form at both promoters on the same DNA molecule. The *trfAp-1* mutation, which reduces melting at *trfAp*, does not stimulate melting at *trbAp*. Therefore, it must be a later stage of transcription initiated at *trfAp* that blocks *trbAp*. The face to face arrangement of the promoters would make it possible for forward transcription from *trfAp* to inhibit *trbAp*. Third, the presence of *korA* changes the balance between *trfAp* and *trbAp* activities by the effect of its product on *trfAp*. Although we expect the main reason for this effect to be the inhibition of *trfAp* transcriptional activity, we note that this switching is not caused by the other *trfAp* repressors KorB and TrbA. This KorA-specific inhibition/stimulation also occurs *in vivo* in the presence of apparently normal levels of other regulators

that act at this region—namely, KorB, KorF, KorG, and TrbA (unpublished data), suggesting that the switch is real rather than an artefact resulting from dissection of the system.

This *trfAp/trbAp* switch located adjacent to *trbA*, whose product represses *trfAp* further, is reminiscent of the immunity regions of temperate phage regulating the selection of lysogenic or lytic cycles. Like temperate phage, propagation of a conjugative plasmid can proceed in two ways. Vegetative replication after activation of *oriV* by the TrfA protein is equivalent to the lysogenic state. Conjugative spread of plasmid molecules, which depends on rolling circle replication initiated at *oriT*, as well as products of the *tra* and *trb* operons, resembles the invasion of a new host as in the lytic cycle. A major goal of this work is to explain and predict plasmid behavior (copy number, stability, and transfer frequency) under different physiological conditions and in a range of species. Identification of the KorA-mediated genetic switch governing genes for these alternative modes of RK2 propagation is a key step in this process.

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