Promoters of the Broad Host Range Plasmid RK2: Analysis of Transcription (Initiation) in Five Species of Gram-Negative Bacteria

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ABSTRACT

A broad host range cloning vector was constructed, suitable for monitoring promoter activity in diverse Gram-negative bacteria. This vector, derived from plasmid RSFl010, utilized the firefly luciferase gene as the reporter, since the assay for its bioluminescent product is sensitive, and measurements can be made without background from the host. Twelve DNA fragments with promoter activity were obtained from broad host range plasmid RK2 and inserted into the RSFl010 derived vector. The relative luciferase activities were determined for these fragments in five species of Gram-negative bacteria. In addition, four promoters were analyzed by primer extension to locate transcriptional start sites in each host. The results show that several of the promoters vary substantially in relative strengths or utilize different transcriptional start sites in different bacteria. Other promoters exhibited similar activities and identical start sites in the five hosts examined.

PLASMID RK2 is a 60-kilobase, self-transmissible member of the IncP group of plasmids, whose hallmark is their broad host range (for a recent review, see SMITH and THOMAS 1989). These plasmids are capable of transfer, replication, and maintenance in most genera of Gram-negative bacteria, Bacteroides and Myxococcus representing the only known exceptions (BRETON, JAOUA and GUESPIN-MICHEL 1985; GUINEY, HASEGAWA and DAVIS 1984).

To be propagated or selected in such a wide variety of hosts, the essential genes of the plasmid must be expressed. These include genes required for replication, stable maintenance, conjugal transfer and antibiotic resistance. RK2 also encodes a group of proteins (kil) (FIGURSKI et al. 1982) which are lethal to cells that have lost the plasmid (for a recent review, see THOMAS and HELINSKI 1989). The transcriptional repressors (kor) of these genes are therefore also essential for the propagation of the plasmid. The mechanisms utilized by the IncP and other broad host range plasmids to ensure controlled expression of their genes in a wide-range of bacteria are not known. This question is of importance to our understanding of the properties of broad host range plasmids since transcription initiation factors are not universally conserved among the Gram-negative bacteria (THOMAS and FRANKLIN 1989).

To begin studying the basis of this versatility in gene expression, promoter cloning vectors were constructed that replicate in diverse Gram-negative bacteria and contain a reporter gene whose product can readily be quantified in the various cellular environments. Specifically, the firefly luciferase gene, lacking a promoter, was inserted downstream of a multicloning site within a derivative of the broad host range plasmid RSFl010, a member of the IncQ plasmid incompatibility group. The bioluminescent product of the firefly luciferase gene in this transcriptional fusion vector can be sensitively and accurately assayed with no background from the host.

Using this system, we cloned 12 DNA fragments with promoter activity from the broad host range plasmid RK2 and measured their activities in five species of Gram-negative bacteria. We also identified the locations of the promoter fragments on the RK2 physical map. Our results indicate that the strengths of these promoters vary widely when measured in five different Gram-negative bacteria. Also, no consistent pattern was observed when comparing relative activities of each promoter within the same host bacterium. Furthermore, we have analyzed the transcriptional start sites for four of these promoters and have found that their initiation sites in the different hosts are not always identical. Based on these results, we conclude that there is no single universal promoter or adaptive mechanism for expression in diverse hosts. Rather, each promoter may have evolved its own unique adaptive strategy.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and microbiological procedures: The Escherichia coli strains and plasmids used in this study are described in the text. Pseudomonas aeruginosa PAO1161, Acinetobacter calcoaceticus BD413, Rhizobium meliloti 102F34 (al) and Agrobacterium tumefaciens A18 are
standard laboratory strains. Standard protocols and recipes were used for transformation (MANIATIS, FRITSCH and SAMBROOK 1982), plasmid DNA extraction (BIRNBOIM and DOLY 1979), conjugal matings (DITTA et al. 1980) and media (SCHMIDHAUSER and HELINSKI 1985). Construction of pAL4000 is described in the legend to Figure 1. pAL37 is a tetracycline-sensitive deletion derivative of RK2, pAL200 is essentially identical to pAL4000 except that it contains a promoterless chloramphenicol resistance (cat) gene (from Tn9) in place of the luciferase gene, _luc_.

Biochemical procedures, RNA isolation and determination of transcriptional start sites: Restriction endonuclease digestions, ligations, and calf-intestinal alkaline phosphatase digestions were performed according to the manufacturer’s specifications. Procedures for nick translation and RNA-DNA dot blot hybridization (SAMBROOK, FRITSCH and MANIATIS 1989) have been described. Total RNA was isolated as described by ZHU and KAPLAN (1985), except for _A. calcoaceticus_, in which case lysozyme at a final concentration of 500 μg/ml was added at 37°C and incubated for 30 min prior to sodium dodecyl sulfate-protease addition. Primer extensions were performed using AMV reverse transcriptase (Boehringer Mannheim) (VIRTS et al. 1988). A 24-base oligonucleotide, complementary to the XbaI-HindIII segment of the pUC19 polylinker, was chemically synthesized and labeled at the 5' end with [γ-32P]ATP or [γ-35S]ATP and polynucleotide kinase for use as the primer in the reverse transcription reactions. The same oligomer, unlabeled, served as the primer for DNA sequencing reactions performed by the dideoxynucleotide chain termination procedure (SANGER, NICKLEN and COULSON 1977).

**Luciferase assays:** Luciferase assays were performed on logarithmically growing cultures of bacteria essentially as described by WOOD and DELUCA (1987) and SUBRAMANI and DE LUCA (1988). Cells (900 μl) (approximately 1 to 3 x 10^6 cells/ml), grown at 30°C, were added to 100 μl of 10X sonication buffer (50% glycerol, 1 M K2HPO4, 10 mg/ml BSA, 20 mM EDTA, pH 7.4) and immediately chilled on ice. The samples were subjected to two cycles of 24 sec of sonication, separated by 5 min for cooling. Samples of 20 μl of the sonicate were then mixed with 200 μl of 10 mM MgCl2, 3.0 mM ATP, 25 mM glycglycine, pH 7.8, in a disposable cuvette designed for the Monolight 2001 luminometer, both of which were purchased from Analytical Luminescence Laboratories of San Diego, California. After insertion of the cuvette into the luminometer a 100-μl solution of 400 μM D-luciferin (Analytical Luminescence) in 25 mM glycglycine, pH 7.8, was injected, and the peak light emission recorded. A standard curve relating light units to luciferase was determined using purified luciferase enzyme (gift of D. VELLUM).

For whole cell (plate) assays, the bacteria were grown on a nitrocellulose filter placed on solid agar media for 18–36 hr at 30°C. The filters were then soaked in a solution of 1 mM luciferin, 100 mM Na-citrate (pH 5.5) for 10 min in the dark, blotted dry and exposed to X-ray film for 5–10 min.

**RESULTS**

Construction and properties of a broad host range cloning vector containing luciferase as a reporter gene: For the analysis of the relative strengths of promoters of the plasmid RK2 in diverse bacteria a broad host range cloning vector derivative of the IncQ plasmid RSF1010 was constructed that contained a promoterless luciferase reporter gene whose product is readily measured in diverse bacteria _[e.g. E. coli, P. aeruginosa, and R. meliloti (DEWET et al. 1985, PALOMARES, DELUCA and HELINSKI 1989)]_ and upstream restriction sites for making transcriptional fusions. The basic plasmid construct, designated pAL4000, has several important features (Figure 1). (1) The RSF1010 replicon can be stably maintained in diverse species. Although RSF1010 (and thus, pAL4000) is not self-transmissible, it is mobilizable by the transfer functions of plasmid RK2 (DITTA et al. 1980). (2) The tetracycline resistance gene (tetA) of RK2 was included in pAL4000, since this gene can be selected in diverse Gram-negative bacteria (SCHMIDHAUSER 1987). (3) pAL4000 contains the multiple cloning site of pUC19 upstream of the reporter gene. (4) The _E. coli rpoC_ rho-independent transcriptional terminator is upstream of the multicloning site to reduce transcription

**FIGURE 1.—Construction of the broad host range promoter cloning vector pAL4000.** K, P, E, B, Bg, S and X refer to sites for restriction endonucleases _KpnI, PstI, EcoRI, BamHI, BglII, SalI, SacI_ respectively. The plasmid was constructed as follows: (1) The luciferase gene (_luc_). was obtained as a _HindIII-SmaI_ fragment from pKW102 (K. WOOD, unpublished results) and inserted into _HindIII_ and EcoRV digested pAL13, a _Tet5_ derivative of pBR322 generated by filling in the BamHI site, and inserting an 8-base pair _BglII_ linker (_5' CAGATCTG_ at the filled in _SalI_ site). pAL13, carrying _luc_, was designated pAL14. (2) An EcoRI fragment from pAD9 carrying a transcriptional terminator (T) was cloned into EcoRI digested pUC19 to produce pAL1. (3) The tetracycline resistance gene (_tet_). was obtained as a _XhoI-Stul_ fragment, from _pTJS37_ (SCHMIDHAUSER and HELINSKI 1985), inserted into EcoRI digested pAL1 to construct pAL6. (4) A complete _HindIII_ and partial _EcoRI_ digest of pAL6 was carried out to isolate the fragment carrying _TET_, _t_, and _pUC19_ polylinker (MCS). The fragment was inserted into pAL14 to construct pAL23. (5) The translation termination fragment (T) (_5' CTAGCTAGCTAG3'_ at the filled in _SalI_ site) was inserted into pAL23 that was digested with _HindIII_ and filled in to construct pAL27. (6) A complete _BglII_ and partial _EcoRI_ digest of pAL27 yielded the segment containing _tet_, _T_, _MCS_, _luc_, and a region of plasmid pBR322 (coordinates of 185–651 bp). This ligated to an RSF1010 derivative deleted of the 0.8-kb _PstI_ fragment with EcoRI and _BglII_ linkers added to the _PstI_ and EcoRI termini, respectively (BAGDASARIAN et al. 1981).
readthrough into luc. (5) A 12-bp linker containing translational stop signals in each reading frame is between the multicloning site and the luc open reading frame (ORF) in pAL4000 to eliminate translational fusions to luc.

An analogous pBR322-based plasmid, pAL20 (Figure 3), was also constructed. This vector carried the chloramphenicol resistance (cat) reporter gene instead of luc to be able to select weak promoter signals directly. It contains the pUC19 polylinker in the same orientation as in pAL4000, so that transfer of a cloned DNA fragment from it to pAL4000 can be a single step cloning procedure.

Luciferase as a quantitative reporter of promoter activity in different Gram-negative bacteria: It was important to validate the use of the luciferase enzyme as a quantitative measure of promoter activity. A standard curve of enzyme vs. light units demonstrated that the assay was linear from 3.0 pg to 1.5 μg of protein, which corresponded to 3 × 10^6 to 1.5 × 10^9 molecules of luciferase (data not shown). The vector pAL4000 without a promoter fragment insert produced 800–1600 light units per 10^6 cells depending on the host, corresponding to 100–200 molecules of luciferase per E. coli cell (5–10 molecules of luciferase per plasmid copy). A sonicate prepared from plasmid free E. coli gave a value of about 60 light units/10^6 cells. This instrumentation background noise was subtracted from all values obtained.

The firefly luc gene product can be quantified by enzyme activity in many prokaryotic and eukaryotic organisms (reviewed by SUBRAMANI and DELUCA 1988), including E. coli (DEWET et al. 1985), P. aeruginosa, and R. meliloti (PALOMARES, DELUCA and HELINSKI 1989). We inserted known E. coli promoters into pAL4000 [i.e., tac, lac, Kan^R (Tn5)] and found that bioluminescence, due to luciferase could also be measured reproducibly in A. calcoaceticus and A. tumefaciens. Comparison of luc-specific RNA, determined by dot blot hybridization, to bioluminescence due to luciferase activity in different Gram-negative bacteria (KINGSBURY and HELINSKI 1973), this selection favors cointegrates between pAL37 and the pAL20 derivatives that occasionally should be formed by recombination between homologous segments in the pBL1 (Rec+) host. There should only be two restriction patterns, differing only by the size of the parental plasmids.

Mapping RK2 promoters by cointegrate formation: To map the cloned fragments relative to the RK2 DNA molecule, cointegrates between a plasmid carrying the insert and a tetracycline sensitive derivative (pAL37) of RK2 were selected, and characterized as follows. E. coli strain TB1 (polA^R, Rec^R, Nal^R) harboring the self-transmissible plasmid pAL37 was transformed with DNA of each of the pAL20-derived plasmids. Transformants were mated with plasmid-free C2110Nal^R (polA^R, Rec^R) and Nal^R, Tet^R, Kan^R exconjugants were selected. Because pAL20 can not replicate in polA (DNA polymerase 1 deficient) bacteria (KINGSBURY and HELINSKI 1973), this selection favors cointegrates between pAL37 and the pAL20 derivatives that occasionally should be formed by recombination between homologous segments in the TB1 (Rec^R) host. There should only be two regions of homology between the two parental plasmids: in the β-lactamase (bla) genes of RK2 and pBR322 (HEFFRON et al. 1979), and the segments from RK2 cloned in pAL20. Cointegrates formed by recombination in bla would have nearly identical restriction digest patterns, differing only by the size of the particular promoter fragment of RK2. Cointegrates formed by recombination within RK2 sequences would yield a unique restriction pattern for each individual promoter fragment inserted into pAL20 (see Figure 4). In addition, the pattern obtained would indicate the direction of transcription.

Using this conjugal mating/cointegrate analysis technique, exconjugants were selected in C2110Nal^R; their frequency was approximately 10^3–10^4-fold lower than exconjugants obtained when pAL37 and pAL200, a Tet^R RSF1010 derivative capable of autonomous replication in the polA recipient, were used. DNA from these cointegrates was analyzed by restriction endonuclease digestion and for each Sau3A insert two restriction patterns were observed (only one pattern was observed for the cointegrate containing...

TABLE 1
Activity of RK2 promoters in five species of Gram-negative bacteria

<table>
<thead>
<tr>
<th>Promoter designation</th>
<th>Promoter (location)</th>
<th><em>E. coli</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>A. calcoaceticus</em></th>
<th><em>R. meliloti</em></th>
<th><em>A. tumefaciens</em></th>
</tr>
</thead>
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<tr>
<td>pAL4000</td>
<td>None</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>4007</td>
<td>tetA</td>
<td>8.0</td>
<td>11.4</td>
<td>6.5</td>
<td>4.0</td>
<td>6.4</td>
</tr>
<tr>
<td>R9</td>
<td>trpA</td>
<td>4.3</td>
<td>2.0</td>
<td>2.1</td>
<td>1.1</td>
<td>2.4</td>
</tr>
<tr>
<td>R11</td>
<td>kcrA</td>
<td>23.0</td>
<td>10.2</td>
<td>10.7</td>
<td>11.3</td>
<td>20.8</td>
</tr>
<tr>
<td>R15</td>
<td>56.9 CCW</td>
<td>6.8</td>
<td>27.2</td>
<td>4.6</td>
<td>2.1</td>
<td>6.6</td>
</tr>
<tr>
<td>BK3</td>
<td>45.8 CCW</td>
<td>1.2</td>
<td>6.2</td>
<td>0.8</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>BK5</td>
<td>16.3 CW</td>
<td>1.3</td>
<td>1.7</td>
<td>1.1</td>
<td>3.5</td>
<td>3.8</td>
</tr>
<tr>
<td>BK10</td>
<td>korA</td>
<td>11.8</td>
<td>9.6</td>
<td>7.8</td>
<td>5.0</td>
<td>7.0</td>
</tr>
<tr>
<td>BL1</td>
<td>fisA</td>
<td>9.4</td>
<td>11.2</td>
<td>2.2</td>
<td>2.1</td>
<td>6.6</td>
</tr>
<tr>
<td>220</td>
<td>48.9 CCW</td>
<td>1.2</td>
<td>11.8</td>
<td>0.8</td>
<td>0.8</td>
<td>2.1</td>
</tr>
<tr>
<td>223</td>
<td>Tra1 P1,2</td>
<td>1.6</td>
<td>4.9</td>
<td>1.1</td>
<td>2.3</td>
<td>3.7</td>
</tr>
<tr>
<td>87</td>
<td>oriT P1</td>
<td>2.7</td>
<td>10.4</td>
<td>1.7</td>
<td>1.6</td>
<td>2.3</td>
</tr>
<tr>
<td>88</td>
<td>oriT P1</td>
<td>3.1</td>
<td>9.3</td>
<td>2.1</td>
<td>8.9</td>
<td>11.6</td>
</tr>
</tbody>
</table>

Activities are presented as a ratio of the number of light units from cells harboring the plasmid of interest to the number of light units expressed by pAL4000 in a given host bacterium. See MATERIALS AND METHODS for assay conditions. CCW and CW refer to counter clockwise and clockwise direction of promoter activity. Each value represents an average of five independent trials; the standard deviation was less than 10% for each value obtained.

pAL20 that does not contain a *Sau*3A insert as expected). Figure 4A, lanes 1–4 and 7–10, shows restriction digestion patterns for eight independently isolated cointegrates. One of the two patterns was similar
for each insert and that obtained for pAL20. Digestion with Apl, ClaI and XhoI restriction enzymes showed that the site of recombination was within the segment of homology present in their bla genes (Figure 4). Cointegrate DNAs formed between pAL37 and pAL20-R15 (Figure 4B, lanes 1–3) or, for example, pAL37 and pAL20-BK3 (Figure 4C, lanes 1–2) also yielded a unique pattern for each promoter. Analysis of these fragment sizes enabled us to map the site of homologous recombination within pAL37 and the relative orientation of the two plasmids to distinguish the direction of transcription within the parent RK2 plasmid. For example, a unique pattern was observed after Apl, ClaI and XhoI digestion of cointegrates between pAL37 and pAL20–220 (Figure 4B, lanes 6–8) which indicated recombination between the promoter segment carried on pAL20–220 and its corresponding region of pAL37. Similar analyses of pAL37 and the remaining Sau3A inserts into pAL20 were performed to yield the data summarized in Figure 5. Some of these insertions (R11, BK10, R9, BL1, 223) corresponded to previously identified RK2 promoters, as subsequently confirmed by either DNA sequencing or restriction mapping. Others (BK3, R15, BK5, 220), however, mapped to areas of RK2 which have not been extensively studied and, therefore, only their relative coordinates on the RK2 map are given.

Promoter activity in five Gram-negative species: Twelve RK2 promoter fragments cloned into pAL20 were isolated as KpnI-PstI restriction fragments and inserted into pAL4000. These plasmids were conjuga
gally transferred into P. aeruginosa, A. calcoaceticus, R. meliloti and A. tumefaciens, using the ColEl-derived helper plasmid pRK2013 (DITTA et al. 1980). The results of luciferase assays are shown in Table 1 and are presented as a ratio of the number of light units obtained for the plasmid of interest compared with the pAL4000 vector in each individual host. It is clear that the strengths of the different promoter segments vary widely within a particular host and that several of the individual promoter segments direct variable levels of luciferase when comparing their relative activities across species lines. Due to post-transcriptional effects that are likely to differ from host to host, the numbers obtained are likely to be valid only when comparisons are carried out within a particular species. For interspecies comparisons, only general trends are likely to be significant.

Three promoters [R11 (ktc1A), BK10 (koIA) and 4007 (tetA)] exhibited moderate to strong activity in all five hosts examined. Three promoters [BK3, 220, 4087 (oriT P61)] showed moderate to strong activity only in P. aeruginosa; the activity was either weak or below the vector background in the other bacteria. Two promoters [4088 (oriT P1) and 223 (Tra1 P1,2)] exhibited moderate or strong activity in P. aeruginosa, R. meliloti and A. tumefaciens, but relatively weak activity in E. coli and A. calcoaceticus. One promoter (R15) was moderate to very strong in all hosts except R. meliloti. The BK5 promoter, on the other hand, showed moderate strength in R. meliloti and A. tumefaciens only, while the BL1 promoter exhibited moderate to strong activity in E. coli, P. aeruginosa and A. tumefaciens, and substantially less activity in A. calcoaceticus and R. meliloti. Finally, the R9 (tnpA) promoter was weak to moderate in strength in all the hosts tested.

Identification of transcriptional starts: Since it has been shown that the sequences which constitute promoters in one species are not necessarily identical to those which constitute promoters in another (see THOMAS and FRANKLIN 1989), we were interested in...
determining the transcription initiation sites for the cloned RK2 promoters in the different hosts. Four promoter fragments (kcrA1, korA, oriT P1 and Tra1 P1.2) were selected for primer extension analysis to identify transcription start sites. Our results for these four promoters indicated that start sites are constant among species with some fragments but are variable for others. Promoter R11 (kcrA1) initiated transcription from the identical three bases in the five bacteria studied (Figure 6A,i); these start sites corresponded to those previously published for E. coli (THOMAS et al. 1988).

The results with the BK10 (korA) promoter showed that transcription was also initiated from the same site in all five hosts (Figure 6A,ii), which coincided with the start site determined in E. coli (THOMAS and SMITH 1986). However, for A. tumefaciens, three smaller species of transcripts additionally were observed, located 44, 47 and 48 bases downstream from the conserved start. These are either the result of the activity of an additional promoter unique for A. tumefaciens, or processing sites for the transcript which initiates upstream.

The results with the oriT P1 promoter (4087) demonstrated that it also contained an initiation site conserved among all five hosts (Figure 6B,iii). However, a unique start site was observed for A. tumefaciens, 29–30 bases upstream. Since this was upstream of the conserved initiation site, it suggested a unique promoter sequence for A. tumefaciens in this region. The A. tumefaciens reverse transcription product which comigrated with those of the other hosts indicates an alternative promoter, or may result from RNA processing of the transcript initiated from the upstream promoter region. In A. calcoaceticus, two faster migrating species, located 81 and 101 bases downstream, respectively, were detected. Each of these bands was of approximately equivalent intensity as the one from the conserved upstream site and by a similar argument could represent either unique A. calcoaceticus promoter(s) or a processing product.

With the Tra1 P1.2 promoter transcription started from one site in E. coli, P. aeruginosa and A. calcoaceticus (Figure 6B,iv), and from a second site (32–33 bases upstream) in R. meliloti and A. tumefaciens. A secondary start or processing site for R. meliloti coincided with the initiation sites for the other three hosts;
Figure 7.—Sequence of the pir-wt and pir-6 promoters and their activities relative to pAL4000 in the five species of Gram-negative bacteria. The location of the mutation is indicated by the arrow.

its intensity was considerably weaker than the upstream site. While the signals for the TraI P2 promoter were somewhat less intense than for the other three promoters, they were reproducible.

A mutation affecting promoter activity in all five hosts: The pir promoter of the narrow host range plasmid R6K directs transcription of the essential replication initiation protein \( \pi \) (Stalker, Kolter and Helinski 1982). We had isolated a promoter-down mutation pir-6, due to a C → A transversion in the −35 region (A. Greener, unpublished results) (Figure 7).

To ascertain whether the pir promoter was functional in the other hosts and whether the change in the −35 region affected its activity to the same degree in each of them, both the pir-WT and pir-6 promoters were inserted into pAL4000. The resulting plasmids, pAL4001 and pAL4002, respectively, were transferred into the different bacteria and luciferase was assayed (Figure 7).

The pir promoter, previously shown to be relatively strong in E. coli (Shaferman et al. 1982), displayed high activity in other hosts except P. aeruginosa. The pir-6 mutation, which resulted in an approximately 2–3-fold weaker promoter in E. coli both in vivo and in

Figure 6.—Analysis of transcriptional start sites. Primer extension analysis of the RNA start sites was carried out for the kerAl [A(i)], kerA, [A(ii)], orfTpR1 [B(iii)], and TraI pl2 [B(iv)] promoters. For each: Lanes 1–5: primer extension reactions on RNA isolated from E. coli, P. aeruginosa, A. calcoaceticus, R. meliloti, and A. tumefaciens, respectively; a \( ^{32} \text{P} \) or \( ^{35} \text{S} \)-24-base oligonucleotide complementary to the XbaI-HindIII segment of the pUC19 polylinker in pAL20 derivatives carrying the promoter fragments was used as the primer which was extended using 20 units of AMV reverse transcriptase. Lanes 6–9: GATC DNA sequencing reactions; the sites where RNA transcripts initiate or are processed are indicated as follows: all hosts; E. coli, P. aeruginosa, A. calcoaceticus, R. meliloti and A. tumefaciens, respectively; a \( ^{37} \text{P} \) or \( ^{35} \text{S} \)-24-base oligonucleotide complementary to the XbaI-HindIII segment of the pUC19 polylinker in pAL20 derivatives carrying the promoter fragments was used as the primer which was extended using 20 units of AMV reverse transcriptase. Lanes 6–9: GATC DNA sequencing reactions; the sites where RNA transcripts initiate or are processed are indicated as follows: all hosts; E. coli, P. aeruginosa, A. calcoaceticus, R. meliloti only; A. tumefaciens only; A. calcoaceticus only; R. meliloti and A. tumefaciens only. The consensus −35 and −10 hexamers corresponding to the E. coli promoter are designated.
vitro (A. Greener, unpublished results) was also weaker in the other hosts. This suggested that the
−35 hexamer of the pir promoter is involved in transcription initiation in all five bacteria examined.

DISCUSSION

Plasmids which have evolved a broad host range have a number of important adaptations that ensure their stable propagation in the wide variety of cellular environments. One of these adaptations is the ability to carry out the controlled expression of its genes in different environments. RNA polymerases from a number of eubacterial species have been purified and found to be structurally related; the core enzymes have a common organization (αββ′) (McClure 1985; Gao and Gussin 1991). The sigma factors of these polymerases, however, which are responsible for the specific selection of transcriptional start sites, vary widely (Thomas and Franklin 1989). Beside the heterogeneity in size and primary amino acid sequence of the σ factors, the specific bases with which they direct their cognate core polymerases to interact are not identical. Examples demonstrating that a promoter from one Gram-negative bacterium is often nonfunctional in another have been documented (reviewed in Thomas and Franklin 1989). However, some promoters examined have exhibited broad host range initiation activity (e.g., the tac promoter; Frey, Midd and Krisch 1988) and a previous study with the trfA promoter in RK2 demonstrated that its start site was conserved between E. coli and two species of Pseudomonads (Pinkney et al. 1986).

We have developed a series of cloning and luc reporter gene expression vectors suitable for selection and quantitation of promoter activity in diverse Gram-negative bacteria. Using these vectors, we analyzed promoters from the broad host range plasmid RK2 to determine how transcription in different hosts is accomplished and compared the relative strengths of twelve RK2 promoters within a particular host bac-terium.

The data presented in Table 1 indicate that no universal rules concerning relative activities of promoters from broad host range promoters can be drawn. A wide variation in their relative activities within each host is observed. Certain regions which act as moderate to strong promoters in one Gram-negative bacterium do not do so in another. It is possible that either the gene products or the RNA synthesized from these variable promoter elements may play a different role in the RK2 broad host range properties and/or are regulated differentially depending upon the host. It is also possible that some of the promoters examined in this study are subject to up- or down-regulation by other genes within the intact RK2 plasmid.

Two promoters, analyzed by luciferase activity and by primer extension mapping, were highly expressed in all hosts studied. The promoter designated R11, which was mapped to the kcrA1 gene by cointegrate formation and confirmed by DNA sequencing, is responsible for producing the KcrA1 protein. It is not known what role this product plays in the RK2 life cycle. However, its promoter structure is recognized similarly in all five hosts investigated on the basis of the identity of the transcriptional start sites (Figure 6A). The identification of the start sites here coincides with a previous study of the kcrA1 promoter in E. coli reported by Thomas et al. 1988.

A second promoter directing substantial luciferase activity in all hosts tested was the korA promoter, responsible for transcribing the korA, incC and korB genes (Thomas and Smith 1986). The KorA and KorB products have been implicated in the control of RK2 copy number via transcriptional repression of the RK2-specified replication gene trfA (Shingler and Thomas 1984). In addition, they prevent synthesis of a number of host lethal gene products (KilA, KilB, KilC, KilD) by binding to their respective promoters (reviewed in Thomas and Helinski 1989). The incC gene product whose coding region overlaps korA (8 bases upstream and produced out of frame) has been implicated in RK2-specified incompatibility (Meyer and Hinds 1982; Thomas and Smith 1986).

The substantial activity of the korA promoter in all hosts is, therefore, not surprising. However, although the transcriptional start sites were identical for all five hosts, an alternative initiation region, representing either a unique promoter or an RNA processing site and located 44–48 bases downstream, was observed for A. tumefaciens. This apparent start downstream appeared relatively strong and could represent the primary start site in vivo in A. tumefaciens. Regardless of the mechanism, the resulting 5′ end of the korA mRNA, unique for A. tumefaciens, is not likely to specify the incC product since it lacks the ribosome binding site. It is possible that an incC product is not required in A. tumefaciens.

The remaining two promoters studied by primer extension (Tra1 pL2 and oriT pR1) exhibited interspecies differences in start site utilization and/or RNA processing. The oriT pR1 promoter, which controls transcription for 15-kDa and 26-kDa proteins that are involved in conjugal transfer (Guiney and Lanka 1989), had identical starts in all five hosts. However, a secondary start site unique to A. tumefaciens was present 29–30 bases upstream of this consensus. In addition, two smaller species of transcripts were observed for A. calcoaceticus starting at 81 and 101 bases downstream respectively. The downstream sites may correspond to alternative initiation sites or RNA proc-
essing in *A. calcoaceticus* and their presence may have regulatory significance for this host.

The pL2 promoter, which may be responsible for transcription of the primase operon and expression of additional proteins involved in conjugal transfer (FURSTE *et al.* 1989), exhibited identical starts among all hosts except *A. tumefaciens*; for this bacterium, a distinct initiation site was located 43–44 bases upstream. This upstream start site was also observed for *R. meliloti* and appeared to be utilized in that host considerably more frequently than the downstream site common for the other three hosts.

It is interesting to note that for the four promoters analyzed by primer extension, all exhibit identical start sites in *E. coli*, *P. aeruginosa* and *A. calcoaceticus*. Although substantial sequence information is not yet available for determining a consensus promoter sequence for *P. aeruginosa* and *A. calcoaceticus*, based on these data, it may be similar to the *E. coli* consensus.

For the remaining eight promoters that were not analyzed by primer extension, each appears somewhat unique with regard to its relative activities in the various Gram-negative bacteria tested. Preliminary results suggested that with one or two exceptions, a given promoter had maximal activity in *P. aeruginosa*. This is not surprising since *P. aeruginosa* was the host from which plasmid RK2 originally was isolated. The one notable exception, BK5, is located downstream from the RK2 replication protein(s) gene *trfA*, and directs transcription of the gene in an antisense orientation.

An interesting observation was also made concerning the activities of a wild-type and mutant promoter isolated from the narrow host range (*E. coli*) plasmid R6K. Even though the relative strength of the *pir* promoter varies, the mutation, a C → A transversion in the −35 region (Figure 7) resulted in an approximately twofold reduction of luciferase activity in the five hosts examined. These data suggested that this particular base and the adjoining bases are involved in the initiation of transcription in all five organisms.

There is a great deal which remains to be learned concerning how the plasmid RK2 ensures its broad host range transcription and achieves the balanced gene expression required for its replication and stable maintenance in the variety of Gram-negative hosts. The results presented here represent an initial attempt at studying some of the parameters of transcription from a broad host range plasmid in Gram-negative bacteria other than *E. coli*. The plasmids designed for this study may be useful in identifying and determining the primary sequence of different classes of functional promoters in a variety of gram-negative bacterial species. The promoter fragments isolated and partially characterized in this study may contain members of some of these different classes. The use of different classes of promoters with differential activity in a range of bacteria may be one of the strategies employed by the broad host range plasmid RK2 to ensure the appropriate level of gene expression for conjugal transfer, stable maintenance and antibiotic resistance in diverse genetic environments.

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**LITERATURE CITED**


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