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Expression of Antibiotic Resistance Genes in the Integrated Cassettes of Integrons

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Plasmids containing cloned integron fragments which differ only with respect to either the sequence of the promoter(s) or the number and order of inserted cassettes were used to examine the expression of resistance genes encoded in integron-associated gene cassettes. All transcripts detected commenced at the common promoter P_{ant}, and alterations in the sequence of P_{ant} affected the level of resistance expressed by cassette genes. When both P_{ant} and the secondary promoter P2 were present, transcription from both promoters was detected. When more than one cassette was present, the position of the cassette in the array influenced the level of antibiotic resistance expressed by the cassette gene. In all cases, the resistance level was highest when the gene was in the first cassette, i.e., closest to P_{ant}, and was reduced to different extents by the presence of individual upstream cassettes. In Northern (RNA) blots, multiple discrete transcripts originating at P_{ant} were detected, and only the longer transcripts contained the distal genes. Together, these data suggest that premature transcription termination occurs within the cassettes. The most abundant transcripts appeared to contain one or more complete cassettes, and is possible that the 59-base elements found at the end of the cassettes (3′ to the coding region) not only function as recombination sites but may also function as transcription terminators.

Integrons are genetic elements that contain the determinants of a site-specific recombination system by means of which they are able to capture genes (21). The captured genes, most commonly antibiotic resistance genes, are part of discrete mobile cassettes which contain the gene coding region and a recombination site, known as a 59-base element, located at the 3′ end of the gene (Fig. 1) (6–8, 10). The 59-base elements are recombination sites recognized by the integron integrase, and cassettes are inserted at *attI* (6), a unique integrase recognition site located in the integron adjacent to the integrase gene (19). Once integrated, cassettes are formally part of the integron, and in naturally occurring integrons there appear to be no restrictions on the number or order of inserted cassettes (see reference 10 for a compilation). Moreover, as the cassettes are discrete units that can be independently mobilized by the integron integrase (7), the arrangement of the cassettes in the insert region can be altered by excision of individual cassettes or reassortment (7, 8), or new cassettes can be precisely inserted (6).

In general, the initiation codons of the genes found in cassettes are located very close to one boundary of the cassette and a promoter is not included in the cassette. The cassettes are all inserted in the same orientation with respect to their coding regions and are believed to be expressed from a common promoter, P_{ant}, which is the only region with strong similarity to the *Escherichia coli* promoter consensus present in the conserved segment of the integron (5′-conserved segment) located 5′ to the cassettes (Fig. 1) (4, 21). This promoter is located 214 bases from the inner boundary of the 5′-conserved segment (Fig. 1) and was first identified by insertional inactivation as that responsible for transcription of the *dhfrII* gene in the plasmid R388 (25). The presence of a promoter in this region was also demonstrated by S1 nuclease mapping of transcripts of the integron-associated *oxa2* gene of R46 (17); however, the location of the transcription start site was not determined accurately in that study. Several of the minor variations in the sequences of the 5′-conserved segment of integrons from different locations are situated in either the −35 or the −10 region of P_{ant} (21), and although these changes might be expected to affect the expression of the cassette genes, the relative strengths of these promoter variants had not been assessed. In a few integrons, namely, In2 from Tn*21* and some of its close relatives, a second promoter is also located in the 5′-conserved segment 119 bases downstream of P_{ant} (Fig. 1). This secondary promoter, P2, has been created by the insertion of three G residues, which increases to 17 the spacing between preexisting −35 and −10 boxes. P2 has been shown to be active (20, 27), but the relative contributions of P_{ant} and P2 to resistance gene expression had not been determined.

Preliminary indications that the level of expression of an integron antibiotic resistance gene may be affected by its position within the insert region came from a previous study (7) of the rearrangement of the inserted gene cassettes. One of the plasmids used, pRMH52, contains a fragment of the integron In4 (found in Tn*1696*) cloned into pACYC184 (Fig. 2A). The In4 fragment includes three complete cassettes in the order *aacC1*-orfE-*aadA2* (Fig. 2A); *aacC1* encodes an aminoglycoside acetyltransferase [AAC(3)-Ia] and confers resistance to gentamicin, *aadA2* encodes an aminoglycoside adenylyltransferase [AAD(3″)] and confers resistance to spectinomycin and streptomycin, and the open reading frame (orfE) has no known function. Though pRMH52 did not confer resistance to concentrations of spectinomycin above 3 mg/ml, plasmid DNA isolated from cells expressing the integrase gave rise at low frequency to transformants that formed colonies on 5 mg of spectinomycin per ml. The simplest explanation for this observation was that the plasmids in these transformants contained two copies of the *aadA2* gene, and in most cases this was so. However, isolates containing only a single copy of *aadA2* were

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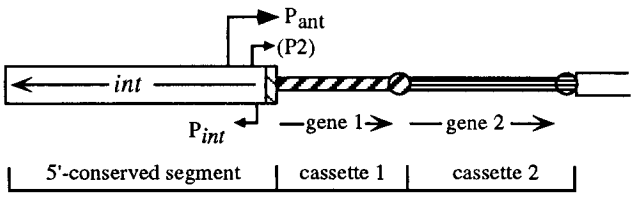

FIG. 1. Locations of promoters in the 5′-conserved segment of integrons. P_{ant} is the common promoter present in all integrons, while P2 is a secondary promoter present in only a few integrons. P_{int} is the promoter for the divergently transcribed integrase gene (*int*). Coding regions of inserted cassettes, designated genes 1 and 2, and of *int* (horizontal arrows), 59-base elements (shaded circles at the 3′ ends of cassettes), and *attI* (19) (hatched box) are indicated.

found. In these, either the *aadA2* cassette had moved to the first position (e.g., pRMH82 in Fig. 2A) or the orfE cassette had been lost (pRMH65 in Fig. 2A), suggesting that the level of spectinomycin resistance may be dependent on the position of the *aadA2* cassette in the array.

In this study, we have examined the general features of expression of cassette genes, including the effects of promoter mutants and variants and of cassette position on expression of the cassette antibiotic resistance genes. A collection of plasmids containing integron fragments cloned in the vector pACYC184 and differing in only one feature (promoter sequence or cassette order) were used. Mutants and natural variants of P_{ant} influenced the level of antibiotic resistance expressed by the cassette genes. Primer extension analysis revealed only transcripts originating at P_{ant}, except when P2 was present and transcripts originating at both promoters were detected. When multiple cassettes were present, the level of resistance was highest when the gene was in the first cassette and was reduced by the presence of preceding cassettes. In Northern blots of RNA from cells containing plasmids with various cassette configurations, several transcripts that originated at P_{ant} but had different lengths were detected. The lengths of the most abundant transcripts indicated that they terminated at or near the 3′ end of individual cassettes, suggesting that the 59-base elements (the cassette-associated recombination sites) may also act as transcription terminators.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* UB5201 is F^- *pro met recA56 gyrA* (9). Plasmids are described in Table 1. pRMH262 was constructed from pRMH232 by *int*-mediated insertion of an *aadA2* cassette that was generated in vitro and introduced into the cells by transformation (6). It was noticed that pRMH262 did not contain the *Hin*cII site that overlaps the −35 region of P_{ant} in the original parent In4, and DNA sequencing of the P_{ant} region revealed that pRMH262 contains a single base mutation such that the sequence of the −35 region of P_{ant} is TAGACA instead of the wild-type TTGACA. The parental plasmid pRMH232 similarly does not contain this *Hin*cII site, and the point mutation presumably arose during construction of pRMH262. pRMH94 consists of a *Hin*cII-*Hin*dIII fragment of pRMH231 cloned between the *Eco*RV and *Hin*dIII sites of pACYC184. In pRMH94, the 5′-conserved segment sequence up to and including the first base of the P_{ant} −35 promoter region is replaced with vector sequence, and hence the −35 promoter sequence is ATGACA. pRMH264, isolated by *int*-mediated deletion of the *aacA4* cassette from pRMH260, has the P_{ant} sequence −35 TGGACA and −10 TAAGCT, perhaps as a result of *recA*-independent homologous recombination with the P_{ant} region from Tn*21*, which is carried on the plasmid expressing the integrase. The sequence of the P_{ant} region of all the above plasmids was determined using RH67 (see below) as the primer. Plasmids that contain rearrangements of the integrated cassettes were all constructed by Int-mediated deletion, rearrangement, or insertion of cassettes as described in previous studies (6–8). Int-mediated events involve precise movement of complete mobile cassettes. In all cases, the cassette content and order were confirmed by detailed restriction mapping, and in many cases the new boundaries were sequenced.

Determination of antibiotic resistance levels. Bacteria were grown to stationary phase in L broth (16) containing chloramphenicol (25 μg/ml) (resistance encoded by pACYC184) with shaking at 37°C and diluted in saline (0.9% [wt/vol] NaCl), and approximately 100 to 300 cells were spread in duplicate on L-agar plates containing a range of concentrations of streptomycin, kanamycin, or gentamicin (Sigma Chemical Co., St. Louis, Mo.). Colonies were counted after 48 h of incubation at 37°C. For each strain and antibiotic, the range of concentrations required was determined empirically. A broad range of antibiotic concentrations was initially employed to find the approximate range within which cells became susceptible to the antibiotic. Subsequent experiments then narrowed down this range to one in which successive dose increments were reflected in gradually decreasing plating efficiencies. The number of cells able to form colonies on agar plates containing each antibiotic concentration was expressed as a percentage of the number of colonies formed on plates containing no antibiotic. The percent survival was then plotted against antibiotic concentration, and the concentration of antibiotic required to achieve 50% loss of cell viability (IC_{50}) was deduced from the graph and taken as a measure of the antibiotic resistance of the strain. For each strain, two determinations were performed with independent cultures and the IC_{50}s were averaged.

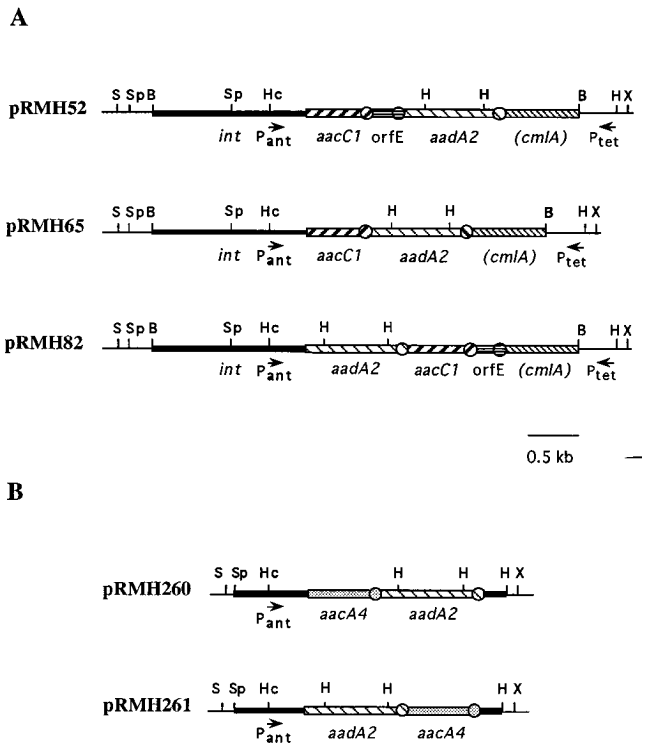

FIG. 2. Structures of rearranged integron derivatives. (A) Plasmids derived from In4 in Tn*1696*. pRMH52 contains a *Bam*HI fragment of In4. pRMH65 and pRMH82 were derived from pRMH52 by integrase-mediated rearrangement (7). (B) Plasmids derived from In6 in pSa. pRMH260 contains an *Sph*I-*Hin*dIII fragment of In6 (6). pRMH261 was generated from pRMH260 by integrase-mediated deletion and reinsertion of the *aadA2* cassette. All fragments are cloned in pACYC184, and the vector sequences are indicated (thin black lines). The integron 5′- and 3′-conserved segments (thick black lines), inserted cassettes (thick shaded lines), and the 59-base element at the 3′ end of each cassette (shaded circle) are shown. Only part of the *cmlA* cassette is included in the cloned fragment in the In4 plasmids. P_{ant} is the common promoter located in the 5′-conserved segment, while P_{tet} is the promoter for the pACYC184 tetracycline resistance gene. Restriction sites: B, *Bam*HI; H, *Hin*dIII; Hc, *Hin*cII; S, *Sal*I; Sp, *Sph*I; X, *Xba*I.

Preparation of RNA and Northern hybridization. RNA was prepared from UB5201 harboring plasmids containing cloned integron fragments by the diethylpyrocarbonate method (24). For Northern hybridization, 10 μg of each RNA sample was heated at 55°C for 15 min in 8% formaldehyde–50% formamide prior to electrophoresis through a 1.4% agarose gel containing 6.5% formaldehyde, using a running buffer containing 100 mM morpholinopropanesulfonic acid, 25 mM sodium acetate, and 0.1 mM EDTA (pH 7). RNA was transferred to a Zeta-Probe membrane (Bio-Rad, Hercules, Calif.) by capillary transfer in 20× SSC (3 M NaCl, 0.3 M trisodium citrate; pH 7). The membrane was cut into strips and hybridized as described previously (7) with probes specific for each cassette and for the 5′-conserved segment. The probes were oligonucleotides RH67 (5′ GCTTGGATGCTCGAGGCATAGAC 3′, the complement of bases

TABLE 1. Plasmids

Plasmid	Description[a]	Relevant phenotype[b]	Source or reference
pACYC184		Cm^r Tc^r	5
pRMH52	3.7-kb *Bam*HI fragment of In4 (Tn*1696*) in pACYC184; cassette order *aacC1*-orfE-*aadA2* (*cmlA*)	Cm^r Gm^r Sp^r Sm^r Int$^+$	7
pRMH58	*Pst*I-*Xba*I fragment of In6 cloned in pSU2718; cassette order *aacA4-aadA2*	Cm^r Gm^r Km^r Sp^r Sm^r	7
pRMH61	Int-mediated loss of *aacC1* and orfE from pRMH52; cassette order *aadA2*-(*cmlA*)	Cm^r Sp^r Sm^r Int$^+$	7
pRMH63	Int-mediated loss of *aadA2* and orfE from pRMH52; cassette order *aacC1*-(*cmlA*)	Cm^r Gm^r Int$^+$	7
pRMH65	Int-mediated loss of orfE from pRMH52; cassette order *aacC1-aadA2*-(*cmlA*)	Cm^r Gm^r Sp^r Sm^r Int$^+$	7
pRMH67	Int-mediated loss of *aadA2* from pRMH58; cassette *aacA4*	Cm^r Gm^r Km^r	7
pRMH71	Int-mediated loss of *aacA4* and *aadA2* from pRMH58; no inserted cassettes	Cm^r	7
pRMH82	Int-mediated rearrangement of pRMH52; cassette order *aadA2-aacC1*-orfE-(*cmlA*)	Cm^r Gm^r Sp^r Sm^r Int$^+$	7
pRMH94	*Hinc*II-*Hin*dIII fragment of pRMH231 in *Eco*RV-*Hin*dIII sites of pACYC184; cassette *aacA4*	Cm^r Km^r	This study
pRMH231	*Sph*I-*Hin*dIII fragment of pRMH67 cloned in pACYC184; cassette *aacA4*	Cm^r Gm^r Km^r	6
pRMH232	*Sph*I-*Hin*dIII fragment of pRMH71 cloned in pACYC184; no inserted cassettes; contains spontaneous mutation in −35 region of P_{ant}	Cm^r	6
pRMH237	Int-mediated insertion of *aadA2* into pRMH94; cassette order *aadA2-aacA4*	Cm^r Gm^r Km^r Sp^r Sm^r	This study
pRMH246	Int-mediated insertion of *aadA1* into pRMH231; cassette order *aacA4-aadA1-aacA4*	Cm^r Gm^r Km^r Sp^r Sm^r	This study
pRMH253	Int-mediated insertion of *aadA1* into pRMH231; cassette order *aadA1-aacA4*	Cm^r Km^r Sp^r Sm^r	This study
pRMH254	Int-mediated insertion of *aadA2*-orfE into pRMH231; cassette order *aadA2*-orfE-*aacA4*	Cm^r Gm^r Km^r Sp^r Sm^r	This study
pRMH255	Int-mediated insertion of *aadA2* into pRMH63; cassette order *aadA2-aacC1*	Cm^r Gm^r Sp^r Sm^r Int$^+$	This study
pRMH260	Int-mediated insertion of *aadA2* into pRMH231; cassette order *aacA4-aadA2*	Cm^r Km^r Sp^r Sm^r	This study
pRMH261	Int-mediated insertion of *aadA2* into pRMH231; cassette order *aadA2-aacA4*	Cm^r Km^r Sp^r Sm^r	This study
pRMH262	Int-mediated insertion of *aadA2* into pRMH232; cassette *aadA2*; contains spontaneous mutation in −35 region of P_{ant}	Cm^r Sp^r Sm^r	This study
pRMH264	Int-mediated deletion of *aacA4* from pRMH260; cassette *aadA2*; contains mutations in −35 and −10 regions of P_{ant}	Cm^r Sp^r Sm^r	This study
pRMH272	*Sph*I-*Hin*dIII fragment of In2 cloned in pACYC184; cassette *aadA1*	Cm^r Sp^r Sm^r	This study
pRMH280	Int-mediated loss of orfE and *aacA4* from pRMH254; cassette *aadA2*	Cm^r Sp^r Sm^r	This study

[a] The *aadA1* and *aadA2* genes each encode an AAD(3″) and confer resistance to streptomycin and spectinomycin; *aacC1* encodes AAC(3)-Ia and confers resistance to gentamicin; *aacA4* encodes AAC(6′)-IIc and confers resistance to gentamicin and kanamycin; *cmlA* confers resistance to chloramphenicol; and orfE confers no known phenotype. Parentheses indicate that only a part of the *cmlA* cassette is present.

[b] Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline.

1352 to 1330 in reference 11) for the 5′-conserved segment, and RH56 (5′ GTCTCCGAGCTCACGACCGAAAAG 3′) for the *aacC1* cassette, RH54 (5′ GGCTAGAAAGCTTTTGGAACAGGGCG 3′) in the orfE cassette, and RH50 (5′ CATCAAGCTTTACGGCCACAGTAACC 3′) in the *aadA2* cassette, which have been described previously (7). The probes were 5′ end labelled with [γ-^{32}P]ATP (Bresatec Ltd.) by using T4 polynucleotide kinase (IBI, New Haven, Conn.).

Determination of mRNA start points. Start points were determined by measuring the size of the product of extension of the primer RH67. RNA from UB5201 containing either pRMH52 or pRMH261 was used as a template. A 10-pmol sample of RH67 was 5′ end labelled as described above and purified by electrophoresis through a 20% acrylamide gel. The labelled primer was annealed with 50 μg of RNA for 2 h at 37°C in 20 mM Tris-HCl (pH 7.5)–200 mM NaCl–1 mM EDTA–0.5% sodium dodecyl sulfate. After ethanol precipitation, the primer was extended with 20 U of avian myeloblastosis virus reverse transcriptase (IBI) in 25 mM Tris-HCl (pH 8)–10 mM $MgCl_2$–10 mM dithiothreitol–0.5 mM deoxynucleoside triphosphates–8 U of RNasin (Promega, Madison, Wis.) at 42°C for 60 min. The reaction was stopped by the addition of EDTA to 20 mM, and RNA was removed by digestion with RNase A (40 μg/ml) (Sigma Chemical Co.). The extension product was analyzed by electrophoresis through a 6% denaturing polyacrylamide gel together with dideoxy sequencing tracks primed with RH67 as markers and visualized by autoradiography.

DNA sequencing. Plasmid DNA for double-stranded sequencing was isolated by using Magic MiniPrep columns (Promega), primers were annealed by the method of Jones and Schofield (14), and the DNA was sequenced with a Sequenase kit (United States Biochemical Corp., Cleveland, Ohio).

RESULTS

Cassettes are transcribed from the promoter(s) in the 5′-conserved segment. That P_{ant} is responsible for the expression of the resistance genes in inserted cassettes was confirmed by determining the level of resistance conferred by plasmids derived from pRMH261 (Fig. 2B) which have mutations in P_{ant} (Table 2). pRMH261 contains two cassettes, *aacA4* and *aadA2*, together with the upstream region containing P_{ant}. The sequence of P_{ant} was determined and consists of a −35 region TTGACA separated by 17 bases from a −10 region TAAACT. P2 is not present. The *aadA2* gene confers resistance to spectinomycin and, to a lower level, to streptomycin, but the concentrations of spectinomycin required to inhibit the growth of cells harboring the *aadA2* gene are impracticably high, and streptomycin was used here. The *aacA4* gene confers resistance to kanamycin and gentamicin (12), and resistance to kanamycin was measured. The level of antibiotic resistance was determined by estimating the concentration of antibiotic required to reduce the plating efficiency to the 50% level (IC_{50}) as described in Materials and Methods. For cells containing pRMH261, the IC_{50} occurred at 1,220 μg of streptomycin per

TABLE 2. Effects of mutations in the P_{ant} promoter on gene expression

Plasmid	Cassette order	Promoter sequence		IC_{50} (μg/ml)[a]	
		−35	−10	SM	KM
pRMH261	*aadA2-aacA4*	-TTGACA-	-TAAACT-	1,220	115
pRMH237	*aadA2-aacA4*	-ATGACA-	-TAAACT-	55	6
pRMH280	*aadA2*	-TTGACA-	-TAAACT-	1,000	NA
pRMH262	*aadA2*	-TAGACA-	-TAAACT-	450	NA
pRMH264	*aadA2*	-TGGACA-	-TAAGCT-	65	NA
pRMH253	*aadA1-aacA4*	-TTGACA-	-TAAACT-	1,100	70
pRMH272[b]	*aadA1*	-TGGACA-	-TAAGCT-	360	NA

[a] IC_{50}, concentration of antibiotic at which 50% of cells plated form viable colonies. SM, streptomycin; KM, kanamycin. NA, not applicable.

[b] P2 (-TTGTTA-[17]-TACAGT-) is also present in pRMH272.

ml and 115 μg of kanamycin per ml (Table 2). Lower levels of resistance were observed in derivatives which have mutations in P_{ant} (Table 2). In pRMH237, the first base of the −35 sequence is altered from a T to an A residue, and this change resulted in a 20-fold decrease in the level of resistance conferred by both cassette genes. In pRMH262, the second base in the P_{ant} −35 sequence is changed from a T to an A residue, and the level of resistance to streptomycin conferred by this plasmid was 2.7-fold lower than for pRMH261 (Table 2). Thus, the integrity of P_{ant} is essential for expression of the cassette antibiotic resistance genes, and the fact that the two insert genes in pRMH237 were equally responsive to the effect of a particular promoter mutation indicates that both genes are transcribed from P_{ant}.

The P_{ant} promoter configuration in the plasmid pRMH264 is found in several naturally occurring integrons and differs at two positions, one in the −10 region and one in the −35 region, from that in pRMH261. Cells containing pRMH264 were resistant to only a level of streptomycin 20-fold lower than for pRMH261, indicating that the promoter strength of these two natural variants differs by at least 20-fold (Table 2). The contribution of the secondary promoter P2 was examined by cloning into pACYC184 a fragment of In2 (from Tn*21*) which contains both the weak version of P_{ant} and P2 preceding the *aadA1* gene. The IC_{50} of streptomycin conferred by the resulting plasmid pRMH272 was 360 μg/ml, which is threefold lower than that conferred by pRMH253, in which the strong P_{ant} variant precedes the *aadA1* gene (Table 2). Thus, while P2 is clearly a stronger promoter than the weak P_{ant} variant, the combined strength of the weak P_{ant} promoter and P2 is less than that of the strong P_{ant} promoter. The AAD(3″) proteins encoded by *aadA1* and *aadA2* share 86% identical amino acids (cf. references 3 and 13), and *aadA1* and *aadA2* confer essentially the same level of streptomycin resistance when expressed from the same promoter (compare pRMH253 and pRMH261 in Table 2).

The start point of the resistance gene transcripts was mapped by extension of a primer located downstream of P_{ant} (complementary to bases 1352 to 1330 in reference 11) with RNA from cells containing pRMH261 or pRMH52 used as a template. The promoter sequence in pRMH52 is identical to that in pRMH261 (27a, 28). The major product (Fig. 3) corresponds to a transcription start at an A residue at position 1223 in the sequence of Hall and Vockler (11), i.e., 8 bases downstream of the P_{ant} −10 sequence. A minor product 1 base longer was also present but is not distinguishable in Fig. 3. Similar results (not shown) were obtained with a primer within the *aadA2* cassette, indicating that there are no further transcription start points between P_{ant} and the beginning of the first cassette. Primer extension using RNA isolated from a strain containing pRMH272 revealed a major product corresponding to transcripts originating at P2 and a minor product corresponding to transcripts originating at P_{ant} (data not shown). This indicates that both the weak P_{ant} variant and P2 are active and confirms that when this promoter configuration is present the majority of transcripts originate at P2.

The level of antibiotic resistance conferred by the *aadA2* gene is influenced by the position of the *aadA2* cassette. The effects of cassette position on gene expression were initially examined by measuring the level of resistance to streptomycin of *E. coli* UB5201 harboring each of the three plasmids pRMH52, pRMH65, and pRMH82, in which the position of the *aadA2* cassette varies (Fig. 2A). Only the strong P_{ant} promoter is present in these plasmids (28). Figure 4A shows the effect of streptomycin on the plating efficiency of cells containing pRMH52, pRMH65, and pRMH82. A sharp decrease in

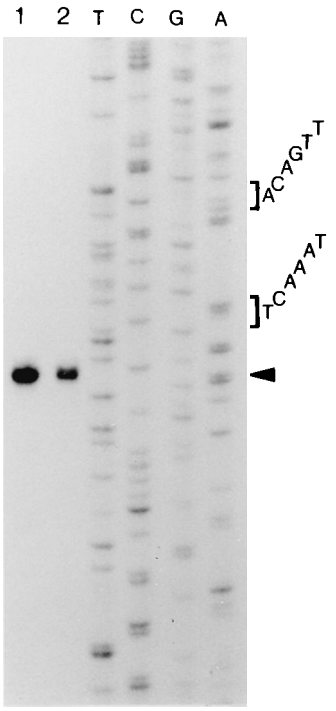

FIG. 3. Mapping of transcription initiation points. Primer RH67 was extended using RNA prepared from *E. coli* UB5201 containing pRMH261 (lane 1) or pRMH52 (lane 2) as a template. The extension products were separated by polyacrylamide gel electrophoresis. Size markers were from sequencing reactions generated from pRMH52 DNA primed with RH67. T-, C-, G-, and A-specific lanes are indicated. The sequence of pRMH261 is identical to that of pRMH52 in this region. The sequences of the P_{ant} −10 and −35 promoter regions are shown at the right, and the 5′ endpoint of the mRNA is indicated (arrowhead). RNA from plasmid-free UB5201 gave no extension product (result not shown).

the survival of cells containing pRMH52 (*aacC1*-orfE-*aadA2*) occurred at relatively low levels of streptomycin, and the plating efficiency was reduced to 50% at a streptomycin concentration of 60 μg/ml. In contrast, for cells containing pRMH82, in which the *aadA2* cassette is relocated to the first position, the IC_{50} of streptomycin was 1,120 μg/ml. pRMH65, which lacks the orfE cassette, conferred an intermediate level of streptomycin resistance, with an IC_{50} of 580 μg/ml. Since the only difference between the three plasmids is the cassette order, these results indicate that the level of expression of the *aadA2* gene is significantly influenced by the position of the cassette in the insert region. That the effects are due to the presence of upstream rather than downstream cassettes can be deduced by comparing pRMH52 and pRMH65; loss of the upstream orfE cassette to form pRMH65 leads to an increase in the resistance conferred by the *aadA2* gene.

The *aadA2* cassette is also found in the integron In6 in plasmid pSa (10, 26). Although the published sequence of the *aadA2* cassette from In6 (26) differs from that of *aadA2* in In4 (compiled from references 2, 7, 22, and 28), the differences represent errors in the In6 sequence (3, 27a). pRMH260 and pRMH261 (Fig. 2B) include the two possible configurations of the *aacA4* and *aadA2* cassettes together with 5′-conserved segment sequences from In6, and the cloned fragments are in the same orientation in pACYC184 as the In4 fragment in pRMH52. The level of resistance to streptomycin was again highest when the *aadA2* cassette was first and was reduced fourfold when *aadA2* followed the *aacA4* cassette (Fig. 4B).

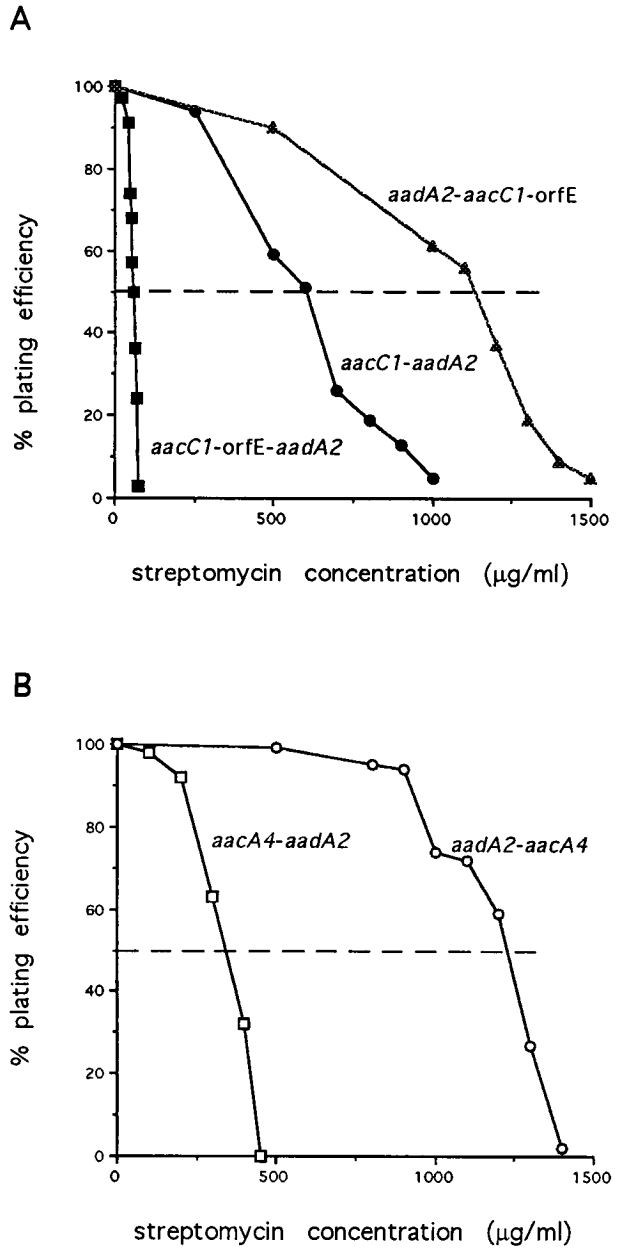

FIG. 4. Effect of increasing streptomycin concentrations on the plating efficiency of *E. coli* UB5201 harboring plasmids containing cloned integron fragments. (A) In4 derivatives. ■, pRMH52; ●, pRMH65; ▲, pRMH82. (B) In6 derivatives. □, pRMH260; ○, pRMH261. Plating efficiency values are the means of two independent determinations. The IC_{50} (50% plating efficiency) is indicated (dashed line).

Effects of position on expression of other cassette genes. A systematic survey of the antibiotic resistance of cells harboring cloned fragments containing various arrangements of the cassettes (Table 3) revealed that the level of expression of other antibiotic resistance genes found in integron-associated cassettes is also affected by the cassette position. In all cases, expression of the cassette gene was greatest when the cassette was promoter proximal and was reduced by the presence of preceding cassettes. For example, for cells harboring a plasmid containing the *aacC1* gene, the IC_{50} for gentamicin was 45 to 55 μg/ml when *aacC1* was in the first cassette (pRMH52 and pRMH65) (Table 2) and was reduced four- to fivefold when the *aacC1* cassette was located behind the *aadA2* cassette (pRMH82). Similar conclusions apply to the *aacA4* and *aadA1* genes.

TABLE 3. Effects of cassette order on resistance levels

Plasmid	Cassette order[a]	IC_{50} (μg/ml)[b]		
		SM	GM	KM
In4 derivatives[c]				
pRMH52	*aacC1*-orfE-*aadA2*-(*cmlA*)	60	45	NA
pRMH65	*aacC1*-*aadA2*-(*cmlA*)	580	55	NA
pRMH82	*aadA2*-*aacC1*-orfE-(*cmlA*)	1,120	11.3	NA
pRMH61	*aadA2*-(*cmlA*)	1,110	NA	NA
pRMH255	*aadA2*-*aacC1*-(*cmlA*)	1,350	10.5	NA
In6 derivatives				
pRMH260	*aacA4*-*aadA2*	310	8.6	170
pRMH261	*aadA2*-*aacA4*	1,220	4.6	115
pRMH231	*aacA4*	NA	ND	165
pRMH246	*aacA4*-*aadA1*-*aacA4*	460	ND	150
pRMH253	*aadA1*-*aacA4*	1,100	ND	70
pRMH254	*aadA2*-orfE-*aacA4*	1,250	4.4	50

[a] Parentheses indicate that only a part of the *cmlA* cassette is present.
[b] IC_{50}, concentration of antibiotic at which 50% of cells plated form colonies. SM, streptomycin; GM, gentamicin; KM, kanamycin; NA, not applicable; ND, not determined.
[c] Contain 19-bp duplication of 5′-conserved segment sequence (28).

Not only was the expression of each resistance gene affected by the presence of one or more upstream cassettes (as described above), but each cassette also reduced the expression of downstream genes (Table 3). The magnitude of the reduction caused by a single upstream cassette was generally two- to fourfold but appears not to depend only on the nature of the cassette, as the effect of a particular upstream cassette on individual downstream genes can differ. For example, an upstream orfE cassette appeared to have a particularly strong effect on the expression of the *aadA2* gene. Resistance was reduced 20-fold when the *aadA2* gene was preceded by both the *aacC1* and the orfE cassettes (pRMH52) but only twofold if the only upstream cassette was *aacC1* (pRMH65), indicating that the net effect of the orfE cassette was an approximately 10-fold reduction. In contrast, when the orfE cassette was located upstream of the *aacA4* gene, kanamycin resistance was reduced only 2.3-fold (Table 3).

Transcripts end within and at the ends of cassettes. As all of the cassette genes are transcribed from the same promoter, the simplest explanation for the observed effects of upstream cassettes on gene expression is either that lower levels of mRNA containing the downstream coding regions are present or that the efficiency of translation is affected by the presence of upstream cassettes. To ascertain whether transcripts are affected, RNA from cells containing plasmids with different insert orders was examined using oligonucleotide probes specific for each cassette and for the 5′-conserved segment. The plasmids used were pRMH52 (cassette order *aacC1*-orfE-*aadA2*), pRMH65 (*aacC1*-*aadA2*), and pRMH82 (*aadA2*-*aacC1*-orfE), and the results are shown in Fig. 5. In most cases, several bands were observed, indicating that discrete transcripts of different lengths were present, and, as the single promoter P_{ant} is responsible for transcription of the inserted cassettes (see above), all transcripts should start at the same point. That there are no major internal promoters is confirmed by the fact that all the transcripts detected with cassette-specific probes were also visualized by the 5′-conserved segment probe. The discrete bands therefore represent transcripts that end at spe-

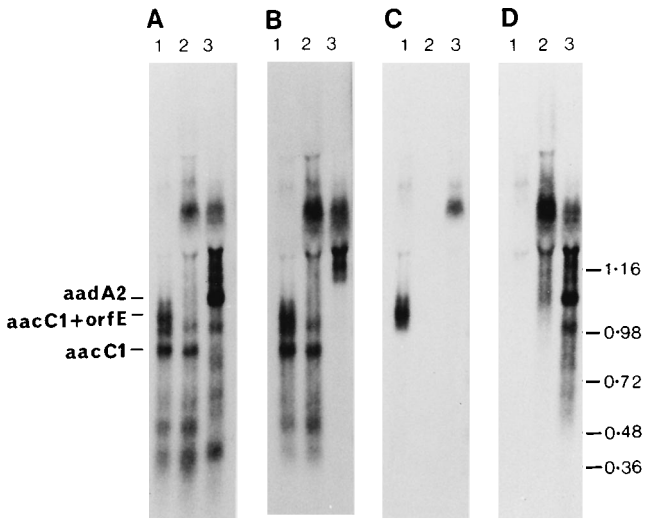

FIG. 5. Northern analysis of transcripts. RNA from cells containing pRMH52 (lanes 1), pRMH65 (lanes 2), or pRMH82 (lanes 3) was separated by electrophoresis, transferred to a membrane, and hybridized with oligonucleotide probes specific for the 5′-conserved segment (A), the *aacC1* cassette (B), the orfE cassette (C), and the *aadA2* cassette (D). The positions of the transcripts ending at or near the 3′ ends of the *aacC1* and the *aacC1* plus orfE cassettes in pRMH52 and the *aadA2* cassette in pRMH82 are indicated on the left. Also apparent are a background smear composed of nascent transcripts and degradation products and artifactual bands at >1.2 kb due to the presence of large amounts of 16S rRNA. Size markers were an *Eco*RI digest of phage SPP-1 end labelled, denatured, and electrophoresed concurrently with the RNA samples. Their positions and sizes (in kilobases) are indicated on the right.

cific sites. These sites all occur within the insert region, since the smallest transcripts observed were approximately 0.4 kb in length (Fig. 5) and in In4 P_{ant} is located 233 bases from the start of the first cassette. For each plasmid, the bands revealed by the probe for the first cassette (Fig. 5B, lanes 1 and 2, and D, lane 3) are the same as those detected by the 5′-conserved segment probe (Fig. 5A). However, only a subset consisting of longer transcripts was detected by probes for the distal cassettes. Thus, for pRMH52 (*aacC1*-orfE-*aadA2*), which expresses the lowest level of streptomycin resistance, only a small proportion of the total transcripts (Fig. 5A, lane 1) was detected by the *aadA2* probe (Fig. 5D, lane 1). On the other hand, for mRNA from cells containing pRMH65 (*aacC1*-*aadA2*), which has lost the orfE cassette and confers a level of streptomycin resistance 10-fold higher than that of pRMH52, a larger proportion of the total transcripts was detected by the *aadA2* probe (cf. lanes 2 in Fig. 5A and D). Because the promoters are identical in these two plasmids, the difference in the proportion of transcripts which contain the *aadA2* gene would translate to a substantial difference in the absolute level of *aadA2*-containing transcripts, and this difference is clearly sufficient to account in large part, if not completely, for the different levels of streptomycin resistance conferred by these two plasmids. Furthermore, since removal of the orfE cassette leads to more transcripts extending into the *aadA2* gene, it seems reasonable to conclude that significant termination of transcripts occurs within the orfE cassette. This conclusion is supported by the presence in mRNA from pRMH52 of the group of strong bands detected by the orfE probe (Fig. 5C, lane 1) but not by the *aadA2* probe (Fig. 5D, lane 1).

In RNA from pRMH52-containing cells, the sizes of the two most intense bands identified with probes specific for both the 5′-conserved segment and the *aacC1* gene approximate the predicted lengths of transcripts originating at P_{ant} and ending at or near the 3′ end of the *aacC1* (0.81 kb) and orfE (1.07 kb) cassettes, respectively (Fig. 5A and B, lanes 1). The larger, but not the smaller, of these two transcripts was also detected with the orfE-specific probe (Fig. 5C). As this probe is complementary to sequence 81 to 105 bases from the 5′ end of the orfE cassette, the smaller transcript cannot extend further than 100 bases into orfE, consistent with an endpoint near the 3′ end of the *aacC1* cassette. Similarly, in RNA from cells containing pRMH82, a major species corresponding in size to a transcript ending close to the 3′ end of the *aadA2* cassette (predicted length, 1.09 kb) was observed (Fig. 5A and D, lanes 3). Thus, transcripts with 3′ termini close to the 3′ ends of the first or second gene cassettes are identifiable. The longer transcripts detected with distal probes (e.g., the *aadA2*-specific probe for pRMH52 and pRMH65) had an electrophoretic mobility greater than that predicted on the basis of their length, presumably because of comigration with rRNA.

In addition to transcripts with endpoints close to the 3′ ends of cassettes, several smaller transcripts were detected with probes for the proximal cassettes, and the calculated length of each of these implicates endpoints at particular sites within the first cassette, *aacC1* in pRMH52 and pRMH65 or *aadA2* in pRMH82. The fact that some transcripts terminate before the end of the first cassette presumably reduces the maximal level of expression of all the cassettes in the insert region, including the first.

DISCUSSION

It has been common to use the level of conferred antibiotic resistance (e.g., MIC) as a criterion in the classification of resistance genes. However, this is not a valid criterion for gene classification, since several factors can influence the level of expression of a particular antibiotic resistance gene. The most obvious are transcription and translation initiation signals, the efficiency of which can readily be altered by mutation. Also, as resistance genes are commonly located on plasmids and as the same gene can be found on different plasmids, the plasmid copy number is a factor. In the case of resistance genes associated with integrons, all of the above factors are likely to play a role. In this study, the version of P_{ant} with the sequence −35 TTGACA and −10 TAAACT found in In4 and In6 was most active, and the variant −35 TGGACA and −10 TAAGCT, which has also been found in several naturally occurring integrons, was 20-fold less active. As the same resistance gene can be found downstream of different promoters in natural integron isolates, the differences in promoter strength can lead to a 20-fold difference in the antibiotic resistance level conferred by the gene. A third promoter configuration found in naturally occurring integrons, e.g., In1 in R46 (11), which is a hybrid of the −10 region (TAAACT) from the strong promoter found in pRMH261 and the −35 region (TGGACA) from the weak promoter in pRMH264, was not examined in this study, but has recently been shown to have an intermediate strength (15). Though the weak version of P_{ant} is found alone in some natural integrons, in In2 (in Tn*21*) it occurs together with a secondary downstream promoter, P2, which compensates for the low activity of the weak P_{ant} promoter, and together they express a level of streptomycin resistance only threefold lower than that of the strong P_{ant} variant. Others have recently measured the relative strengths of the promoter variants by using a chloramphenicol acetyltransferase assay, and the results are substantially in agreement with those obtained in this study using antibiotic resistance measurements (15).

Expression of resistance genes in the inserted cassettes of

integrons is also influenced by the position of the cassette in the insert region. The levels of expression of the cassette genes in the plasmids used in this study are directly comparable, as the insert regions together with the conserved flanking sequence including P_{ant} were cloned into the same vector, pACYC184, eliminating copy number effects. Transcription from the *tet* promoter of pACYC184 (23), which is present in the In4 derivatives (Fig. 2), should not contribute to expression of the cassette antibiotic resistance genes, as the orientation of these genes is opposite to that of the tetracycline resistance gene. The level of antibiotic resistance conferred by any particular cassette gene was highest when the gene was in the cassette located closest to P_{ant} and was reduced when the cassette was situated downstream of one or more other cassettes, the precise level of antibiotic resistance being dependent on both the number and the nature of the upstream cassettes. Analysis of transcripts from a group of three plasmids differing only in the number and order of gene cassettes suggested that these effects were mediated in large part, if not completely, at the level of transcription.

Transcripts which have discrete 3′ ends might arise either by premature termination of transcription or by posttranscriptional processing. Degradation of a primary transcript from the 3′ end by exonucleases can lead to rapid decay of a message and is an important determinant of the lifetime of mRNA species (1), and stem-loop structures in the RNA such as those formed by repetitive extragenic palindromic (REP) sequences have been shown to stabilize upstream RNA by acting as blocks to this degradation (18). Though the analysis of cassette transcripts alone cannot distinguish between premature termination of transcripts and protection of upstream mRNA from degradation, the resistance levels conferred by genes in different cassette arrangements (Table 3) argue against the latter mechanism. If transcripts from an upstream gene were protected from degradation by the secondary structure of the downstream RNA, expression of the promoter-proximal gene would be expected to depend on the number and nature of downstream cassettes. In fact, the resistance levels of genes in the first position appear to be unaffected by downstream cassettes (Table 3). Thus, the effects observed in this study are unlikely to be due to barriers to RNase processing from the 3′ end of a primary transcript. The alternative explanation for the discrete 3′ endpoints of transcripts is that the nascent mRNA chains are terminated at particular sites during synthesis. This explanation is consistent both with the presence of discrete transcripts and with the observed decrease in antibiotic resistance level when upstream cassettes are present.

The estimated lengths of several of the major species suggest that their 3′ ends coincide approximately with the 3′ ends of cassettes, and it appears likely that some feature of the 3′ ends of cassettes affects transcript size. Possible candidates are the recombination sites or 59-base elements located at the 3′ end of the coding region of each gene. These 59-base elements (4, 8, 10) are imperfect inverted repeats and therefore have the potential to form stem-loop structures once transcribed into RNA. Moreover, although 59-base elements constitute a loosely related family, they differ from one another both in sequence and in length (8, 10), consistent with the fact that different cassettes appear to modulate the expression of downstream genes to different extents. Further studies are clearly required to address this issue.

The effects of differences in promoter strength and of cassette position on the level of expression of antibiotic resistance genes are likely to be relevant in the clinical context. In particular, it is possible that the presence of genes which at best confer only modest or low levels of resistance to an antibiotic could fail to be detected if their expression level is lowered sufficiently by either a weak promoter or the presence of upstream cassettes or both. If cassette position is a factor, such silent genes could be disclosed under selective pressure of the antibiotic by integrase-mediated rearrangement of the cassettes to create variants in which the cassette is closer to P_{ant} and thus expressed at higher levels.

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