Transcription of genes involved in the earliest steps of actinorhodin biosynthesis in *Streptomyces coelicolor*

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ABSTRACT

A 170bp long *Bam*HI-*Sau*3A DNA fragment from the *act*III-*act*I intergenic region of the actinorhodin (Act) biosynthetic gene cluster of *Streptomyces coelicolor* A3(2) contains two promoters directing transcription in a divergent manner. One of them, the *act*III promoter, is responsable for the transcription of the *act*III gene and the other controls transcription of the adjacent *act*I region in the opposite direction. Weak activity of the *act*III promoter can be detected in *Streptomyces lividans* and *Bacillus subtilis* in the absence but not in the presence of glucose. Neither promoter seems to function in *Escherichia coli*.

INTRODUCTION

The use of alternative sigma factors is known to be widespread among bacteria (1); the existence of holoenzymes containing alternative sigma factors transcribing different sets of promoters in a cascade mode of action is a central feature of the control of the developmental cycle that ends in spore formation in *Bacillus subtilis* (2). The heterogeneity of the *Streptomyces coelicolor* RNA polymerase is now well documented (3): promoters recognized by different holoenzymes are involved in transcription of the *dag*A gene (4) and the *gal* operon (5), and the product of the *whi*G gene is a new sigma factor controlling the onset of sporulation in aerial hyphae (6). Equivalence between some sigma factors from *S.coelicolor* and *B.subtilis* has been reported (6,7) and there is evidence for the evolution of some sigma factors to control unrelated sets of genes in different bacteria (as could be the case for the sigma factors related to the *Escherichia coli* sigma 54 (1)) involving the *B.subtilis* sigma D and the *S.coelicolor* WhiG factor (6).

To gain further insight into the possible use of holoenzymes from different bacteria to recognize *in vivo* potentially diverse promoters controlling the expression of secondary metabolism genes, the *act*III-*act*I region controlling early steps in the biosynthetic pathway of the antibiotic actinorhodin, a model secondary metabolite from *S.coelicolor* (8), was chosen as a source of promoters. The DNA sequence of the *act*III gene is known, its promoter is identified (9) and the *act*I and the *act*III regions are known to be adjacent on the genome (8). A promoter cloning approach allowed us to map accurately the start point of transcription for the *act*I region as well as to determine whether the promoters for both regions that function in *S.coelicolor* would also work *in vivo* in the closely related bacterium *S.lividans*, in the distantly related low G+C Gram-positive *B.subtilis*, or in the far more distantly related Gram-negative bacterium *E.coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids

S.lividans TK21 (10) was the host for the high copy number promoter-probe vector pIJ486 (11). *B.subtilis* MB11 (*lys*-3, *met*-10, *his*H2) (12) was the host for the high copy number promoter-probe plasmid pPCT2. pPCT2 is a slightly modified version of pPL703 (13), containing, downstream of the *cat*86 gene, the transcription terminators T1 and T2 from the *E.coli rrn*B gene (14); it was originally obtained from J.C.Alonso. *S.coelicolor* M145 (10) containing the plasmid pIJ2303 (which carries the entire *act* cluster on a low copy number vector) (8) was used as the Act$^+$ strain being a higher *act* RNA producer than the parental M145 strain. pIJ2363 is a derivative of pBR329 (15) that contains the *act*I and *act*III region of the *act* cluster (restriction sites 13−17: 8). pMV400 derives from pUC19 (16) and contains a *Sph*I fragment (Fig. 4) spanning the *act*I, VII and IV regions of the *act* cluster (8). pPLc28actIII is a derivative of pPLc28 (17) that carries the *act*III gene (sites 13 to 14: 8). pRM1cat (R.P.Mellado, unpublished) is a pBR322 (18) derivative that contains the *cat* gene and the transcription termination sequences from pCPP-3 (19) downstream of the pUC19 polylinker (16). *E.coli* strains K12Δ H1Δ*trp* (17) and NM514 (*hfl*A, *hsd*R_k) (20) were used for the propagation of pPLc28 actIII and pRM1cat, respectively.

Standard media and manipulations

Procedures for the growth and manipulation of *Streptomyces* and general recombinant DNA manipulation were as described elsewhere (10, 21). Competent *B.subtilis* MB11 and competent *E.coli* were obtained and transformed as described (22 and 23, respectively).

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In vivo transcription

RNA was isolated from vegetative (up to 48 h) and stationary (from 70 h. on) phases of *Streptomyces* cultures grown at 29°C in Yeme medium (10) with or without glucose (1% w/v) as indicated, the mycelium being harvested and the RNA prepared as described before (24). *E.coli* and *B.subtilis* cultures were mid-log phase, grown at 37°C in LB, and the RNA was prepared as described previously (25).

The separated strands of the indicated DNA fragment uniquely labelled at the 5' end were gel-purified, mixed with 50 μg of total RNA, denatured and hybridised at 55°C (26). S1 nuclease digestion was then carried out as described (21, 25). The size values given for the S1 nuclease protected fragments were corrected for the 1.5nt differential migration compared with the sequence ladder (27).

To determine the extent of transcription, sandwich hybridisation experiments (28, 29) were carried out using as a probe the same labelled DNA strand as in the S1 nuclease mapping. Hybridisation conditions were as in the S1 nuclease mapping experiments, except that 200 μg of total RNA were used.

RESULTS AND DISCUSSION

Promoter cloning

DNA from the 1035bp long *act*III region (9; sites 13−14 of ref. 8) cleaved from pIJ2363 was digested with *Sau*3A; the produced fragments were randomly inserted in the *Bam*HI site of pPCT2 (Fig. 1) and *B.subtilis* MB11 was transformed with the resulting ligation mixture. The selected recombinant plasmid pVMb31, conferring Cm^R (5 μg/ml) to the bacteria, contains the *Bam*HI-*Sau*3A fragment (positions 2 to 172) of the *act*III DNA sequence (9) that includes the *act*III promoter, but inserted in the opposite orientation, so that transcription of the *cat* gene took place from a putative '*act*I' promoter in the opposite direction to that of the *act*III gene. The *Eco*RI-*Bam*HI fragment containing the promoter activity from pVMb31 was inserted in the *Streptomyces* plasmid pIJ486 (Fig. 1) and introduced into *S.lividans* to generate pVMs33, in which transcription of the Km^R gene could take place from the *act*III promoter. The *Sma*I-*Bam*HI fragment from pVMb31 was inserted into pIJ486, through its *Bam*HI site and blunt-ended *Hin*dIII site, to generate pVMs31, where the transcription of the Km^R gene could now take place from the cloned fragment in a direction opposite to that in pVMs33.

To place the pPCT2 *cat* gene under the control of the *act*III promoter in *B.subtilis*, the *Pst*I-*Bam*HI fragment from pVMs31 was transferred to pPCT2 via its *Bam*HI and *Pst*I sites, generating pVMb33. For tests in *E.coli*, the 170bp long *Bam*HI-*Sau*3A fragment was retrieved from pVMb31 and cloned in the two possible orientations in pRM1cat through its unique *Bam*HI site (Fig. 1), so that the *cat* gene could be transcribed either from the *act*III promoter (pVMc33) or from the putative '*act*I' promoter driving transcription in the opposite direction (pVMc31).

Very little resistance to the respective reporter antibiotic was conferred by the recombinant plasmids on the different hosts, ranging from virtually negligible in *E.coli* to very low levels (5−10 μg/ml) in *S.lividans* or *B.subtilis*—slightly over the background resistance conferred by their respective vectors—

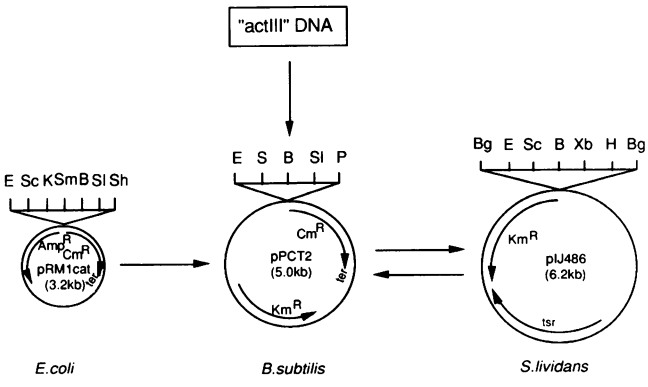

Figure 1. Promoter cloning. The vectors used as promoter-probes for the different bacteria are shown. Amp^R, ampicillin resistance gene; Cm^R, chloramphenicol resistance gene; Km^R, kanamycin resistance gene; tsr, thiostrepton resistance gene. Only unique restriction sites are indicated in the three plasmids. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I; Sc, *Sac*I; Sh, *Sph*I; Sl, *Sal*I; Xb, *Xba*I. A non-indicated *Pst*I site between the *Xba*I and *Hin*dIII sites in pIJ486 (11) was also used in the cloning. See text for details.

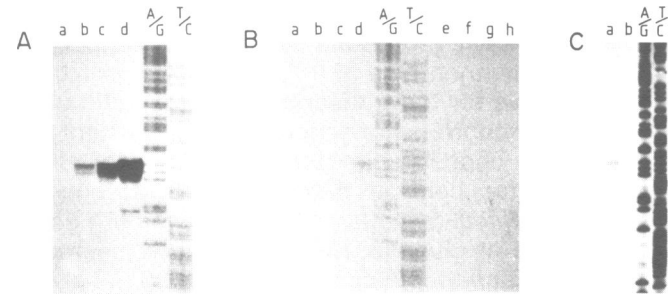

Figure 2. High resolution S1 nuclease mapping of *in vivo* transcription from the *act*III promoter. A. Time course of transcription taking place in *S.coelicolor* (pIJ2303) cultures after 40h. (a), 48h (b), 70h (c) and 85h. (d) of incubation. Blue pigmented antibiotic production took place from 72h of incubation. B. Time course of transcription taking place in *S.lividans* (pVMs33) at the same times as in A, in the presence (e−h) or in the absence (a−d) of glucose in the growth medium. C. Transcription taking place in *B.subtilis* (pVMb33) (a) and *E.coli* (pVMc33) (b). S1 nuclease mapping was performed as indicated in Materials and Methods. A+G (A/G) and T+G (T/G) reactions (29) from the 5' ends labelled single stranded DNA probe were run in parallel as sequence ladders. The existence of more than one protected band might be due to the previously found S1 nuclease nibbling effect (31, 32). Transcription was considered to start at the position marked by the largest protected fragment. Electrophoresis was in 6% acrylamide /8M urea gels.

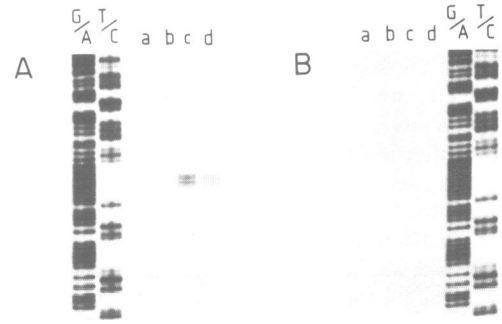

Figure 3. High resolution S1 nuclease mapping of *in vivo* transcription from the *act*I promoter. Time course of transcription taking place in *S.coelicolor* (pIJ2303) (A) or *S.lividans* (pVMs31) (B). Cultures after 48h. (a), 72h. (b), 96h. (c) and 120h. of incubation. See the legend of figure 2 for details of the S1 nuclease mapping procedures. Electrophoresis was in 6% acrylamide/8M urea gels.

when estimated on nutrient broth agar plates. The sequence of the cloned DNA fragments was in all cases confirmed by the chemical degradation method (30).

Transcription initiation

The direction of transcription and the transcription initiation sites were determined by high resolution S1 nuclease protection experiments, with total RNA from the different bacteria harbouring the recombinant plasmids. Transcription initiating within the 170bp *Bam*HI-*Sau*3A fragment from the putative '*act*I' promoter and proceeding towards the reporter antibiotic resistance gene (plasmids pVMb31, s31 and c31) was mapped using the same single stranded DNA fragment labelled at its *Bam*HI 5' end as a probe. Transcription starting within this DNA fragment, but under the control of the *act*III promoter (plasmids pVMb33, s33, and c33), was mapped using the complementary single strand labelled at its *Sau*3A 5' end as a probe. The results are shown in figures 2 and 3.

Transcription from the *act*III promoter initiates at 98 nucleotides from the 5' labelled *Sau*3A end in *S.coelicolor* (Fig. 2A) as previously found using a different probe (9). Transcription from this promoter was time-dependent, coinciding essentially with the production of actinorhodin (blue pigment) by the culture (Fig. 2A, a−d). Transcription from this promoter fell below the limits of detection when cloned in *S.lividans* (Fig. 2B, e−h) as previously found in promoter-probe experiments (9), and it was weakly but reproducibly detected when the cells were grown in the absence of glucose (Fig. 2B, a−d). Again the transcription was time-dependent, as in *S.coelicolor*, indicating the need of effector(s) modulating transcription from this promoter in both bacteria, a result in accord with the activation of the *act*III promoter in *S.lividans* by the presence of the cloned *act*II region (9). Transcription from the *act*III promoter in *S.coelicolor* does not seem to depend on the presence or absence of glucose in the growth medium (result not shown).

Transcription from the cloned *act*III promoter was also very weakly detected in *B.subtilis* when the cells were propagated in the absence of glucose (Fig. 2C, a), and undetected when glucose was present (not shown). No activity of the *act*III promoter was found in *E.coli*, either in the absence (Fig. 2C, b) or in the presence of glucose (not shown).

The putative '*act*I' promoter seems to initiate RNA synthesis in *S.coelicolor* at 95 nucleotides from the 5' labelled *Bam*HI end of the 170bp long *Bam*HI -*Sau*3A fragment, also in a time-dependent fashion (Fig. 3A, a−d), with no dependence on glucose (not shown). The putative '*act*I' promoter does not seem to work in *S.lividans* at any chosen time , either in the presence (Fig. 3B, a−d) or in the absence of glucose (not shown)

The DNA probe was not protected at all from S1 nuclease digestion when RNA from *B.subtilis* or *E.coli* cells carrying the putative '*act*I' promoter was used in the S1 nuclease mapping experiments, either in the presence or in the absence of glucose (not shown). Thus the low activity shown by these promoters during the selection procedure correlates with the difficulty in detecting their respective transcription initiation site by S1 nuclease mapping.

Sandwich hybridisation

To confirm that the transcription mediated by the putative '*act*I' promoter does in fact proceed through the *act*I region in the *S.coelicolor* genome and to check whether or not transcription of this region is initiated upstream of this promoter, a sandwich hybridisation experiment was performed. Total RNA from the actinorhodin-producing *S.coelicolor* strain was hybridised to DNA fragments, previously transferred to nitrocellulose filters, that included the transcription start site and extended over the *act*I region and beyond, in one direction; and over the *act*III region located upstream of the start site, in the other direction. After a first round of hybridisation, the filters were washed and set to hybridise with the same probe used in the S1 nuclease mapping experiments. In theory only the DNA bands containing sequences complementary either to the probe itself or to RNA molecules that can hybridise with the probe, would be revealed; no DNA fragment located upstream of the sequences complementary to the probe should give a positive result unless transcription initiates upstream of the mapped sites. An explanatory scheme is depicted in figure 4. DNA fragments

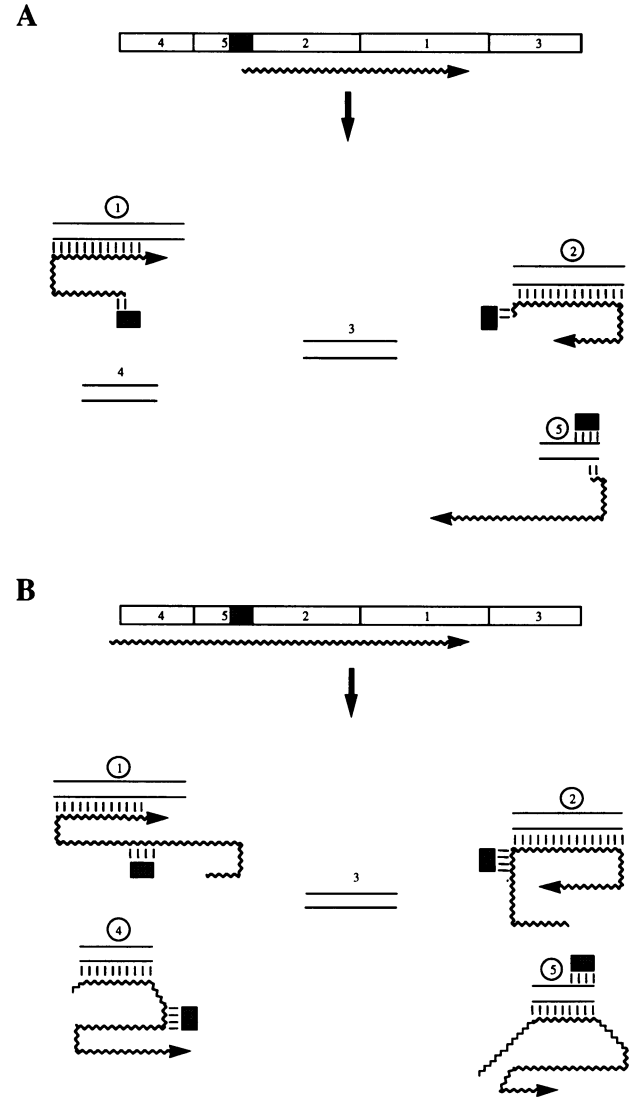

Figure.4. Sandwich hybridisation. The hybridisation resulting from transcription initiating at (A) or upstream of (B) the mapped site is depicted. Numbered boxes are the different DNA fragments produced by endonuclease digestion. The direction and extent of transcription is indicated by a weavy line ended by an arrow. The probe is the dark box included in the DNA fragment number 5. DNA fragments revealed after the second round of hybridisation are indicated by numbered circles. The two strands of each DNA fragment are shown. See text for details.

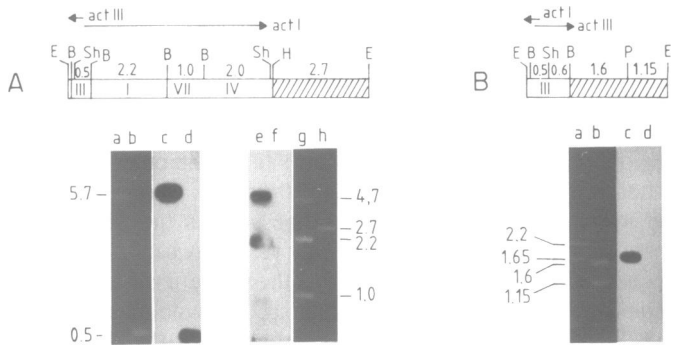

Figure 5. Transcription directed by the *act*I promoter comprises the *act*I region. The physical maps of the plasmids used in the experiments are depicted in the upper part of the figure. All DNA fragments used in the experiments were individually purified. A. DNA from pMV400 was restricted with *Bam*HI or double restricted with *Eco*RI and *Hin*dIII. Fragments containing sequences complementary to the probe were run separately, as the 5.7kb *Eco*RI-*Hin*dIII fragment containing the whole insert (a) or the 0.5kb *Bam*HI fragment (b). The rest of the *Bam*HI fragments from the inserted DNA (g) or the *Eco*RI-*Hin*dIII fragment containing only the vector DNA sequences (h) were also run separately. B. DNA from pPLc28actIII was double restricted with *Sph*I and *Pst*I (a) and the pPLc28 vector DNA was double restricted with *Eco*RI and *Pst*I (b) and run as a negative control. The resulting fragments from the endonuclease digestions were fractionated on 1% agarose gel, transferred to nitrocellulose filters, hybridised in a first round to total RNA from *S.coelicolor* (extracted at the antibiotic producing time) and to the 5′ end-labelled single stranded probe in a second round (28). Hybridisation conditions were the same as in the S1 nuclease mapping experiments. A. Hybridisation results for fragments in lanes a, b, g and h are shown in lanes c, d, e and f, respectively. B. Hybridisation results for fragments in lanes a and b are shown in lanes c and d, respectively. Roman numerals in the rectangular boxes correspond to *act* region present in the cloned fragments; vertical lines within the boxes indicate the extent of a particular *act* region. Dashes boxes indicate vector plasmid DNA. Arrows indicate the direction and extent of transcription from the *act*I and *act*III promoters within the cloned DNA. Figures above the boxes and at the side of the lanes are fragments sizes in kb. Symbols for the restriction endonucleases are given in the legend to figure 1.

containing sequences complementary to the probe, carried as a positive control did hybridise as expected (Fig. 5A, a−d; 5B, a and c). DNA fragments extending over the *act*I region and beyond gave also a positive result (Fig. 5A, e−h), in accord with the genetic data predicting the existence of a polycistronic transcript comprising the *act*I, VII and IV region (8). No hybridisation was detected to a DNA fragment containing sequences located upstream of the probe (Fig. 5B, a and c). Negative hybridisation resulted when DNA fragments containing the vector sequences were used (Fig. 5A f and h; 5B, b and d).

The result of a sandwich hybridisation experiment is always more qualitative than quantitative and some differences on the relative intensities of the revealed bands are to be expected. Even so, the hybridisation to the 1kb *Bam*HI fragment (Fig. 5A, e) although distinguishable over the background (Fig. 5A, f) is weaker than expected, something that also occurs when other partially overlapping DNA fragments are used (not shown). Apart from the continuous transcription comprising the *act*I to IV regions, the existence of internal transcription proceeding in the same direction can not be rouled out. Thus transcription initiating and terminating within the 1kb *Bam*HI fragment might compete with the polycistronic transcript for the same DNA sequences in the first round of hybridisation, resulting in a weaker hybridisation of the polycistronic mRNA to the probe in the second round. Regulation of transcription taking place in the *act*VII region as well as in the whole *act* cluster is currently under investigation. In any case it can be reasonably concluded from

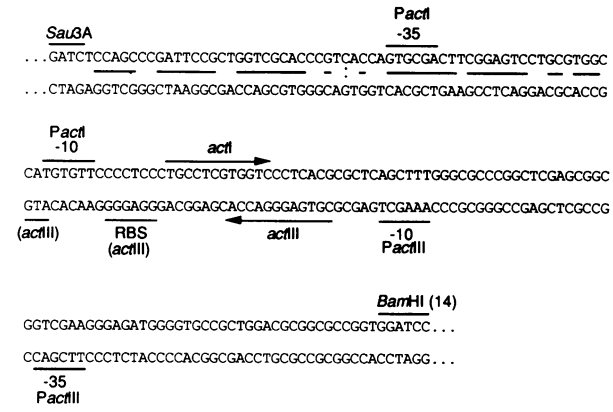

Figure 6. The *act*III-*act*I intergenic region. The DNA sequence of the 170bp long *Bam*HI-*Sau*3A fragment is depicted. The putative −10 and −35 regions are indicated. Arrows indicate the initiation sites and the direction of transcription. The putative ribosome binding site (RBS) for the *act*III gene (9) is indicated. The putative translation initiation triplet is underlined. The number in brackets defines restriction site 14 of Ref. 8. A region of dyad symmetry is indicated by lines between the DNA strands (see Fig. 7).

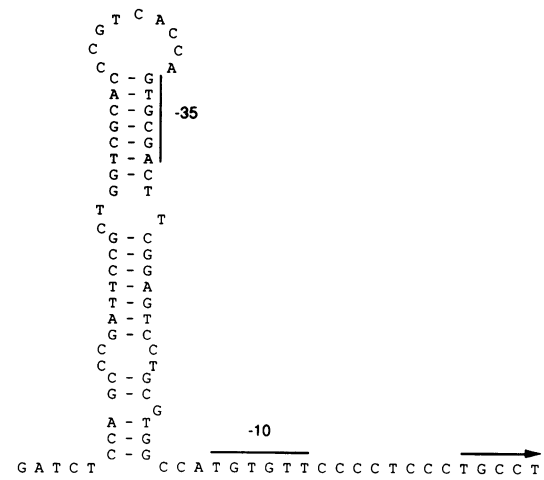

Figure 7. Possible secondary structure in the *act*I-*act*III promoter region. The −10 and −35 sequences for the *act*I promoter are indicated. The arrow indicates the initiation point and direction of transcription (see also figure 6).

the above experiment that there is no detectable transcription initiating upstream of the mapped site for the *act*I region and that the transcription started there comprises at least the whole of the region.

The *act*I-*act*III promoter region

Figure 6 shows the sequence of the 170bp long DNA fragment containing the *act*I and *act*III promoters and the sites where divergent transcription initiates. Comparison of the putative promoter sequences with those of other *Streptomyces* promoters allows a tentative alignment of the *act*III promoter to the *S.coelicolor gal*p2 (5) and *dag*Ap2 (4) promoters (the sequence AAGCTGA being present at the −10 region in both cases). The *act*I promoter could be also tentatively aligned to the *S.coelicolor gal*p1 (TGTGTT and TATGTT sequences at the −10 region, respectively (5)) and it might be one of those *Streptomyces* promoters where the spacing between the −10 and −35

consensus sequences is thought be longer than 17–18 nucleotides (5, 33).

Activity of the *act*III promoter in *B.subtilis* might be due to the existence of an unidentified yet specific sigma subunit equivalent to that of *S.coelicolor* (4); to the existence of a positive regulator (34) with an equivalent mode of action to that of the *act* cluster; or to a somewhat inefficient promoter recognition by one of the *B.subtilis* vegetative holoenzymes (1). It is difficult to assess whether *act*III promoter activity is itself specifically repressed by glucose in *S.lividans*, and perhaps in *B.subtilis*, or whether synthesis of a putative positive regulator is the real target for glucose repression. Experiments to investigate this question are now being carried out.

The absence of detectable activity of the *act*I promoter in *S.lividans*, *B.subtilis* and *E.coli* could be due either to lack of an equivalent RNA polymerase to that of *S.coelicolor*, which is thought to recognize promoters with a longer spacing between the −10 and −35 regions (5, 33); or to lack of an appropriated activator (34), perhaps specific to the *S.coelicolor act* cluster. On the other hand, a potential (cruciform) secondary structure (35) could be predicted to form within the 170bp long *Bam*HI-*Sau*3A fragment (Fig. 7), including the putative −35 region of the *act*I promoter. Cruciform DNA structures have been shown to exist intracellularly (35) and a synthetic promoter to be repressed by extrusion of a cruciform structure in *E.coli* (36). The formation of this cruciform structure involving complementarity within the same DNA strand, might make the promoter inaccessible to the RNA polymerase and also impede progression of *act*III transcription. The action of a putative regulatory molecule interacting with the cruciform structure might be needed in order for both regions to be transcribed. A positive regulator of this kind has also been suggested for the *S.lividans tip*A promoter, which is also included in a cruciform structure (37). The positive regulator might or might not coincide with that in the *act*II region of the cluster (9), since the presence of an activator cloned from another streptomycete restores *act*I and *act*III promoter activities in *S.lividans* (N.M.Romero, V.Parro, M.Rajavel, F.Malpartida and R.P.Mellado, manuscript in preparation). This heterologous activator shares no homology with the one included in the *act*II region (M.A.Fernández-Moreno, J.L.Caballero, D.A.Hopwood and F.Malpartida, submitted). These facts, together with the finding that switching on of the *act*I and *act*III promoters is always subject to temporal control both in *S.lividans* and in *S.coelicolor*, not only indicates the existence of a common, specific mechanism of regulation for both promoters (34), but also suggests the possible existence of more than one positive regulator activating sets of promoters in a cascade mode of action as an overall strategy of controlling gene expression in *Streptomyces* secondary metabolism, in a similar manner to the control of sporulation in *B.subtilis* by sigma factors (2). It is interesting and perhaps surprising that genes involved in different steps of assembly of the polyketide chain of actinorhodin—*act*I coding for the condensing enzyme and acyl carrier protein, and *act*III for the ketoreductase—should be transcribed from promoters of apparently different types with the possibility of differential regulation.

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