Transcription analysis of the *Streptomyces coelicolor* A3(2) *rrnA* operon

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Transcription start sites and processing sites of the *Streptomyces coelicolor* A3(2) *rrnA* operon have been investigated by a combination of *in vivo* and *in vitro* transcription analyses. The data from these approaches are consistent with the existence of four *in vivo* transcription sites, corresponding to the promoters P1–P4. The transcription start sites are located at −597, −416, −334 and −254 relative to the start of the 16S rRNA gene. Two putative processing sites were identified, one of which is similar to a sequence reported earlier in *S. coelicolor* and other eubacteria. The P1 promoter is likely to be recognized by the RNA polymerase holoenzyme containing σ^AhBD^, the principal sigma factor in *S. coelicolor*. P2 also shares homology with the consensus for vegetative promoters, but has a sequence overlapping the consensus −35 region that is also present in the −35 regions of P3 and P4. The −35 sequence common to P2, P3 and P4 is not similar to any other known consensus promoter sequence. In fast-growing mycelium, P2 appears to be the most frequently used promoter. Transcription from all of the *rrnA* promoters decreased during the transition from exponential to stationary phase, although transcription from P1 and P2 ceased several hours before that from P3 and P4.

Keywords: *Streptomyces coelicolor*, ribosomal RNA, transcription, promoter, *rrnA*

INTRODUCTION

Streptomyces, like all members of the order Actinomycetales, have a complex life cycle that involves three stages of differentiation. These morphological changes are accompanied by a wide range of physiological events including the production of secondary metabolites, many of which have activity as antibiotics (Chater, 1989). The molecular processes regulating these events are presently only superficially understood.

Regulation of transcription of stable RNA operons is a pivotal process in all organisms. The only *Streptomyces* rRNA operon for which the transcription has been studied (*Streptomyces coelicolor* *rrnD*; Baylis & Bibb, 1988) contains four promoters. Little is known about the regulation of rDNA transcription in *Streptomyces* and how this relates to differentiation processes. The most studied regulatory phenomenon is stringent control, elicited by accumulation of highly phosphorylated guanosine molecules as a response to starvation conditions. Although ppGpp has been implicated in secondary metabolism (Ochi, 1986, 1987), recent reports have failed to confirm this (Bascaran et al., 1991; Strauch et al., 1991). An intriguing observation was described by Granozzi et al. (1990), who reported a second round of stable RNA production on solid medium that coincided with the development of aerial hyphae.

In contrast to *Streptomyces*, transcription of *Escherichia coli* rRNA operons occurs from two promoters, P1 and P2. For *rrnB*, P1 is tightly regulated whereas P2 is used much less frequently than P1 during exponential growth (Gourse et al., 1986). Recently, expression of all seven *E. coli* *rrn* operons was compared (Condon et al., 1992). Although during fast growth hardly any difference could

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Abbreviation: SCDA, specific catechol dioxygenase activity.
The GenBank accession number for the sequence reported in this paper is X60515.
be detected in transcription of these operons, slight but significant differences were observed in response to some stress conditions, including nutritional shift-down.

Recently, we described the cloning of three of the six rRNA operons of \textit{S. coelicolor} (\textit{rrn}A, \textit{rrn}C and \textit{rrn}E) and sequence analysis of the \textit{rrn}A 16S rRNA gene and upstream region (van Wezel \textit{et al.}, 1991). The nucleotide sequences of the upstream regions of the \textit{S. coelicolor} ribosomal RNA operons \textit{rrn}A and \textit{rrn}D differ significantly, and this prompted us to investigate the locations and relative strengths of the \textit{rrn}A promoters and compare the results with those obtained for the \textit{rrn}D promoters. Here we analyse the relative contribution of each of the \textit{rrn}A promoters to the total transcription of this operon under various growth conditions.

**METHODS**

**Bacterial strains, plasmids and bacteriophages.** \textit{S. coelicolor} M145 was cultivated in Tryptone Soya Broth plus 10% (w/v) sucrose (TSBS), liquid minimal medium (Hopwood \textit{et al.}, 1985), liquid minimal medium supplemented with Casamino acids [designated SMM (Takano \textit{et al.}, 1993)], or on R2YE or minimal agar plates (Hopwood \textit{et al.}, 1985). Liquid cultures were inoculated with spores at a density of 5 x 10<sup>6</sup> c.f.u. ml<sup>-1</sup> and grown at 30°C with vigorous shaking (300 r.p.m.), resulting in reproducibly dispersed growth. In SMM, nitrogen limitation results in a rapid transition into stationary phase approximately 18 h after inoculation of the cultures (Strauch \textit{et al.}, 1991; Takano \textit{et al.}, 1993). RNA used for nuclease S1 mapping was isolated from SMM inoculated with spores germinated for 6 h. The RNA we used for nuclease S1 mapping experiments was isolated by Eriko Takano (John Innes Centre, UK) as described earlier (Takano \textit{et al.}, 1993).

RNA used for primer-extension experiments was isolated from liquid minimal medium cultures inoculated with ungerminated spores. In this case, stationary phase was reached after approximately 33 h. pJ4083 (Clayton & Bibb, 1990a) was used in the promoter-probing experiments. \textit{E. coli} JM109 (Yanisch-Perron \textit{et al.}, 1985) was the host for pUC18 and pUC19, and for bacteriophages M13 mp18 and mp19 (Yanisch-Perron \textit{et al.}, 1985), and was cultivated in LB medium (8 g Difco Bacto-tryptone, 5 g NaCl and 5 g Difco yeast extract per litre).

**In vitro DNA manipulations.** Transformation of \textit{S. coelicolor}, and isolation and cloning of \textit{Streptomyces} DNA, were carried out according to Hopwood \textit{et al.} (1985). Transformation of \textit{E. coli}, and DNA cloning, isolation and gel electrophoresis were performed by standard procedures (Sambrook \textit{et al.}, 1989).

**DNA sequencing.** This was performed using the T7 DNA polymerase sequencing kit (Pharmacia) or Sequenase version 2.0 (USBiochemical). The primers used for sequencing were the same as those used for primer extension. The location of the primers is shown in Fig. 1a.

**RNA isolation.** RNA was isolated from \textit{S. coelicolor} M145 according to Hopwood \textit{et al.} (1985). To remove residual DNA, the RNA was salt-precipitated twice in 0.3 M sodium acetate (pH 6.0). No DNase treatment was applied (DNA was not detectable on agarose gels).

**Promoter-probe experiments.** \textit{xylE} from \textit{Pseudomonas putida} was used as a reporter gene (Zukowski \textit{et al.}, 1983) to assay for \textit{in vivo} promoter activity. Fragments containing different segments of the \textit{rrn}A upstream region were cloned into the poly cloning site upstream of the promoterless \textit{xylE} gene of the vector pBluescript II and introduced into the appropriate \textit{S. coelicolor} hosts by transformation of protoplasts.

\textit{S. coelicolor} transformants containing the correct construct were grown in TSBS plus thioestrepton (5 µg ml<sup>-1</sup>) until actinorhodin production was observed, typically after 40 h of growth. S30 extracts were prepared from these cultures as follows. Mycelium was spun down, washed twice and resuspended in 100 mM phosphate buffer (pH 7.2) followed by sixty 5 s pulses of ultrasound with intermittent pauses of 5 s to allow cooling of the samples. This was followed by a 15 min centrifugation at 30000 g to remove cell debris.

Expression of \textit{xylE} was measured as the rate of catechol conversion by the \textit{xylE} gene product, catechol 2,3-dioxygenase (EC 1.13.11.2). Catechol conversion rates were measured by the increase in absorbance at 375 nm (\textit{A}<sub>375</sub>) after addition of 0.2 mM catechol to diluted S30 extracts. From this, the specific catechol dioxygenase activity (SCDA) was determined as the increase in \textit{A}<sub>375</sub> per min corrected for plasmid copy number and protein concentration. The correction for plasmid copy number was performed as follows. DNA was isolated, a dilution series was prepared, and spotted onto Hybond-N filters (Amersham). These filters were hybridized with a probe recognizing the vector DNA [1 kb BstI fragment containing the \textit{tr} gene; hybridization conditions as described by van Wezel \textit{et al.} (1991)], washed and counted in a Betascope radioactivity counter (Pharmacia). From these data, the relative plasmid copy numbers of the various cultures were determined and used to correct the data obtained from the catechol dioxygenase assay. Only slight differences in copy number were observed: the values fluctuated within a margin of approximately 25%. Protein concentrations were estimated by measuring the \textit{A}<sub>280</sub>. Using these values, all samples were diluted to yield approximately equal protein concentrations.

**Primer extensions with reverse transcriptase.** End-labelling of oligonucleotides and primer extensions with reverse transcriptase was performed as described earlier (Stern \textit{et al.}, 1988). The experiments were carried out at least twice to establish reproducibility. The primers used in these experiments are deoxyoligonucleotides gw-1 (−1 to +18), gw-3 (−430 to −413) and gw-5 (−214 to −198) (Fig. 1a). One microgram of RNA and 1 ng of ³²P end-labelled primer were used in each experiment.

**Southern hybridization with oligonucleotides.** DNA samples were electrophoresed through 0.7% agarose gels in TAE and transferred to Hybond-N, using 20 x SSC as the blotting buffer. Hybridization was carried out in 6 x SSC/0.1% SDS/0.1% pyrophosphate/5 x Denhardt’s solution with 50 µg salmon sperm DNA/ml. Hybridization temperature was derived from the equation \( T_h = T_m - 5 {^\circ}C \), and was 47 °C for deoxyoligonucleotide gw-1, and 49 °C for deoxyoligonucleotides gw-2 and gw-5. Filters were washed in 6 x SSC plus 0.1% SDS at a temperature 3 °C below \( T_m \), with several changes of wash buffer until background was sufficiently reduced.

**Nuclease S1 mapping.** Hybridization of 5 µg RNA with the appropriate DNA probe was performed according to Murray (1986) in NA7CA buffer (Summerton \textit{et al.}, 1983). All subsequent steps were carried out as described by Gramajo \textit{et al.} (1993), under conditions of probe excess.

**In vitro transcription analysis.** Partially purified RNA polymerase holoenzyme preparations were isolated as described by Buttner & Brown (1985). In \textit{in vitro} run-off transcription experiments were performed as described earlier (Buttner & Brown, 1985). Products were analysed on denaturing 6% (w/v) polyacrylamide gels using ³²P end-labelled \( HpaII \) fragments of pBR322 as size markers.
RESULTS

Promoter-probing of the rRNA upstream region

To determine the approximate location of the rRNA promoters and to obtain a rough estimate of their relative strengths, we transformed S. coelicolor M145 with derivatives of the multicopy vector pIJ4083 in which a variety of rRNA upstream fragments has been cloned in front of the promoterless xylE gene. The origin of these fragments is shown in Fig. 1(b). Transformants were grown in TSBS and SCDA levels determined (Table 1). Transcriptional activity increased with the length of the fragments. As can be deduced from the table, promoters are likely to be present between -318 and -199, between -416 and -318 and between -494 and -416.

Repeated attempts to subclone fragments containing the FspI-EcoRI (-708 to -494) part of the rRNA upstream region into pIJ4083 were unsuccessful. The occurrence of multiple copies of this fragment may be lethal to S. coelicolor M145. Perhaps it contains a binding site for a factor essential for vegetative growth. Interestingly, a protein with a binding site in the -708 to -494 region, which is centred around the BglII site at -651 (see Fig. 1a), has been detected and its identity is presently being investigated (van Wezel, 1994).

Specificity of oligonucleotides

Three deoxyoligonucleotides, gvw-1 (-1 to +18), gvw-5 (-214 to -198) and gvw-3 (-430 to -413), were used for primer extension experiments, the results of which are given in the next section. Although optimum conditions for Southern hybridization and DNA-RNA hybridization in primer-extension experiments differ, BamHI- and SalI-digested chromosomal DNA were probed in triplicate with 32P end-labelled oligonucleotides (Fig. 2), to assess the ability of these primers to recognize transcripts originating from the other rRNA operons. As expected, gvw-1 recognizes the 5' end of the 16S rRNA gene in all six rRNA operons, and therefore signals resulting from primer extension using this oligonucleotide will be a mixture derived from all these six rRNA operons. However, since all rRNA transcripts are processed at the same positions (Baylis & Bibb, 1988), the bands arising from primer extension with gvw-1 should reveal the processing sites of rRNA in addition to those of the other rRNA operons. Gvw-3 is operon-specific as it hybridized only with a BamHI and a SalI fragment corresponding to rRNA (Fig. 2). Gvw-5 hybridized with bands corresponding to rRNA, but also to an 18 kb BamHI fragment and a 40 kb SalI fragment. The latter signal does not seem to correspond to an rRNA operon, since all rRNA operons are located on SalI fragments larger than 58 kb (van Wezel et al., 1991). The origin of this band is unclear.

Fig. 1. Nucleotide sequence of the region upstream of the 16S rRNA of rRNA and location of restriction fragments and oligonucleotides. (a) Nucleotide sequence of the rRNA upstream region. The sequence of the 16S rRNA gene is shown in italics. Transcriptional start sites were determined by primer extension (Fig. 3), and are represented by dots. (b) Restriction fragments used for promoter-probing experiments (below), in vitro transcription (top, without asterisk) and nuclease S1 mapping (top, asterisk shows 5' 32P end-label). Abbreviations of restriction enzymes: A, AccI; Ah, AhalI; Ap, ApalI; Bg, BglII; B, BstUI; E, EcoRI; F, FspI; H, Hincll; N, Nael; R, Rsal. Not all restriction sites are shown. Putative transcription start sites are represented by an asterisk.

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Table 1. Promoter-probing of the *rrnA* upstream region

<table>
<thead>
<tr>
<th>Segment of <em>rrnA</em> upstream region</th>
<th>SCDA</th>
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<tbody>
<tr>
<td>No insert</td>
<td>ND</td>
</tr>
<tr>
<td>*RsaI-*Aci</td>
<td>-199 to -6</td>
</tr>
<tr>
<td>*BstUI-*Aci</td>
<td>-318 to -6</td>
</tr>
<tr>
<td>*AbaI-*Aci</td>
<td>-416 to -6</td>
</tr>
<tr>
<td>*EcoRI-*Aci</td>
<td>-494 to -6</td>
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Specific catechol dioxygenase activity (SCDA) values are the mean of three independent experiments. The *BstUI-*Aci construct resulted in a catechol conversion rate measured as a defined as 1.0. ND, No catechol dioxygenase activity detected.

Fig. 2. Southern hybridization of *S. coelicolor* A3(2) chromosomal DNA with the oligonucleotides used for primer extensions. Digestions were carried out with *BamHI* (left panel) and *SalI* (right panel). Southern hybridizations were performed using the oligonucleotides indicated above the lanes. Operon classification is shown on the left and right side of the figure. Lane M shows a DNA size marker, *HindIII*-digested *λ* DNA, in kb.

Location of potential *rrnA* start and processing sites by primer-extension

To locate the potential transcription start and processing sites precisely, primer-extension experiments were performed on RNA isolated from cultures grown in liquid minimal medium (without addition of Casamino acids). *S. coelicolor* M145 mycelium was harvested at different times (12, 22, 29 and 42 h after inoculation with ungerminated spores) and RNA was isolated. Under these growth conditions, stationary phase was reached after approximately 33 h. The oligonucleotides gvw-1, gvw-3 and gvw-5 were used as primers to locate RNA 5' ends resulting from either transcription initiation or processing in the region between the 16S rRNA gene and the upstream open reading frame which ends at nt -653 (van Wezel et al., 1991). From the work published by Baylis & Bibb (1988), it follows that the region downstream of -200 is essential for correct processing of *rrnA* transcripts of all six rRNA operons. Secondly, as shown below, no *in vitro* transcription start sites have been observed for *rrnA* in this region, a result confirmed by the promoter-probe experiments. Therefore, we can assume that RNA end-points correspond to positions upstream of -200 are very likely to have arisen from *in vivo* transcription initiation, and those corresponding to positions between -200 and -1 from processing of the various rRNA precursors.

Processing sites were identified by primer-extension using gvw-1 (-1 to +18, see Fig. 1a). Three RNA end-points were identified, corresponding to nt positions -168, -142 and -89, and referred to as bands I, II and III, respectively (Fig. 3a). The relative intensities of the three bands, as estimated by scanning the autoradiograms, were invariant in all four RNA preparations.

Upon extension of oligonucleotide gvw-5 (-214 to -198) with reverse transcriptase, six major RNA end-points were found (bands C–H in Fig. 3b), located at nt positions -416, -334, -308, -254, -248 and -245, respectively. Bands G, D and F correspond closely to sites of transcription initiation *in vitro* (P2, P3 and P4, respectively; see below). Since gvw-5 also hybridizes to a band that does not correspond to rrnA (Fig. 2), it is possible that at least one of the bands E, G and H observed in Fig. 3(b) reflects non-rrnA-derived transcripts.

To analyse whether transcription initiated upstream of nt position -400, oligonucleotide gvw-3 (-430 to -413) was used for primer extension. Two bands were observed, corresponding to RNA end-points at positions -597 (band B) and -640 (band A) (Fig. 3c). The size of band B is consistent with transcription initiation at P1 (see below). Band A is very strong in lane 1 (12 h), but less intense in lanes 2 (22 h) and 3 (29 h), and strong again in lane 4 (42 h), though weaker than in lane 1. This unexpected (but reproducible) growth-phase-dependence raises the question as to the origin of band A. If it corresponds to a real transcription start site, then the corresponding promoter appears to be utilized with almost equal efficiency in early exponential-phase and in stationary-phase cultures.

Location of transcription end-points by nuclease S1 mapping

RNA was isolated at different time-points, between 9 (OD₄₅₀ 0.3, corresponding to early exponential phase) and 33 h (OD₄₅₀ 2.1, stationary phase) after inoculation of SMM with pregerminated spores. The *NaeI-*HincII (-672 to -151) fragment, ³²P end-labelled at the HincII site (Fig. 1b), was used as a probe. As can be seen in Fig.
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Fig. 3. Location of potential transcription start and processing sites by primer-extension using oligonucleotides gwv-1 (a), gwv-5 (b) and gwv-3 (c). Lanes 1–4: RNA isolated 12, 22, 29 and 42 h, respectively, after inoculation with ungerminated spores. Values to the left indicate the ends of the extension products with respect to the start of the mature 16S rRNA (Fig. 1a). Lanes G, A, T, C: nucleotide sequence determined with the same oligonucleotides as also used for primer extension.

Seven protected bands were observed. Band F corresponds to full-length protection of the probe and may be due to DNA–DNA hybridization, a phenomenon that occurs regularly in nuclease S1 experiments. Bands P1–P4 correspond to protected fragments of approximately 450, 265, 185 and 105 nt, respectively, and are consistent with the possible sites of transcription initiation identified by primer extension, namely those starting at nt positions −597, −416, −334 and −254. Since it was difficult to determine the exact position of the transcription start site of the putative P2 promoter, we performed a nuclease S1 mapping experiment combined with Maxam and Gilbert sequencing using the BamHI–BglII (−609 to −274) fragment as a probe, which indicated that the position shown in Fig. 6 is the most likely start site for P2 (data not shown).

Additional bands were found at positions R1 (approximately 100 nt in length) and R2 (less than 60 nt). These bands probably reflect incomplete hybridization of RNA derived from rrnB–F to the rrnA probe. The bands around position R2 displayed a similar growth-phase-dependence to P3 and P4. Surprisingly, band R1 was only visible late in growth: it became apparent approximately 14 h after inoculation (corresponding to transition phase) and was present throughout stationary phase. It is tempting to suggest that this is due to the expression of a particular rrn operon late in growth, although we cannot rule out the possibility that band R1 originated from an additional (fifth) rrnA promoter (see Discussion).

In vitro transcription analysis

In vitro run-off transcriptions were performed to confirm the location of the rrnA promoters, which were identified by the in vivo transcription analyses described above, and to get an idea of which RNA polymerase holoenzymes are involved in the recognition of these promoters. Run-off transcriptions were performed using fractions of S. coelicolor RNA polymerase that had been partially purified.
the 377 bp BgII fragment and the 502 bp EcoRI–AccI fragment (including 14 bp of the pUC18 multiple cloning site sequence) with RNA polymerase fraction 28 (Fig. 5b).

Run-off transcription using the BgII fragment generated three major transcripts of approximately 325, 145 and 65 nt. These correspond to the putative transcription start sites for P1, P2 and P3 already identified with the FspI–HincII fragment, located at nt positions −597, −416 and −334, respectively. The faint band F presumably represents end-to-end transcription.

In vitro transcription with the EcoRI–AccI template also gave four bands. The transcripts of 425 nt, 345 nt and 265 nt correspond to the putative transcription start sites for P2, P3 and P4 located at nt positions −416, −334 and −254, respectively. Band X does not correspond to any of the transcripts produced by in vitro transcription of the FspI–HincII or BgII fragments, and may therefore be the result of artificial initiation of the RNA polymerase.

**DISCUSSION**

**Putative processing sites**

Primer extension with oligonucleotide gvw-1 revealed an RNA endpoint between −169 and −168 (band I in Fig. 3a). Transcripts from the S. coelicolor rRNA operon are processed at exactly the same position by RNaseIII (Baylis & Bibb, 1988) and, since the sequence of the rRNA operon between −200 and −1 is identical to that of rRNA (van Wezel et al., 1991), it is extremely likely that this represents the major processing site for the rRNA precursors. This is in accordance with the consensus found for the major processing site in other Gram-positive bacteria (Ogasawara et al., 1983; Taschke & Hermann, 1986), including S. ambofaciens (Pernodet et al., 1989). Furthermore, no transcription initiation was detected downstream of nt position −200 when the EcoRI–AccI (−494 to −6) fragment was used as a template for in vitro run-off transcription with S. coelicolor RNA polymerases. Interestingly, an RNA end-point was also detected between the adenosines at positions −90 and −89 (band III in Fig. 3a) and occurs at the base of a computer-predicted stem-loop structure (Baylis & Bibb, 1988). The end-point is positioned exactly opposite the cleavage site that results in mature 16S rRNA. This suggests a mechanism with at least three maturation steps, resulting primarily in a precursor with a 168 nt leader, which is then further processed via an 89 nt leader into mature 16S rRNA. The physiological relevance of the RNA end-point between positions −143 and −142 (band II in Fig. 3a) remains unclear. Since all six rRNA operons are processed in the same way [no comparable band was found by nuclease S1 mapping (Baylis & Bibb, 1988)], it is likely that band II results from an artificial stop of the reverse transcriptase in vitro.

**Identification of the transcription start sites of rRNA**

The promoter-probing experiments predicted the presence of promoters in the regions −494 to −416, −416 to −318 and −318 to −199. These predictions appear to

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**Fig. 4. Location of transcription end-points by nuclease S1 mapping.** The probe used was the 557 nt FspI–HincII fragment (see Fig. 1). Bands P1–P4 represent promoter transcripts; band F indicates full length protection of the probe; R1 and R2 are bands that could not be ascribed to rRNA transcription starts. Lane M, DNA size markers (HpaII-digested pBR322) in nt; the other lanes are labeled with time in hours after inoculation with germinated spores.
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**Fig. 5.** *In vitro* transcription analysis of *rrnA*. Lane M, DNA size markers (*HpaII*-digested pBR322) in bp. RNAP, different fractions of RNA polymerase from a Superose-6 FPLC column. Bands P1–P4 indicate *in vitro* run-off transcriptions originating at each of the promoters. (a) *In vitro* transcription using the *FspI*-HindII (−708 to −151) fragment as a template. F indicates end-to-end transcription of the RNA polymerase. (b) *In vitro* transcription using the *BgII* (−651 to −274) and the *EcoRI*-Accl (−494 to −6) fragments as templates, using RNAP fraction 28. F’ indicates end-to-end transcription; band X was probably the result of artificial initiation of the RNA polymerase.

have been fulfilled by the identification, by *in vitro* transcription, primer extension and nuclease S1 mapping, of three promoters in the corresponding parts of the *rrnA* upstream region: P2 in the −494 to −416 region, P3 in the −416 to −318 region and P4 in the −318 to −199 region (see below).

Eight RNA end-points were observed by primer extension with reverse transcriptase in the region upstream of nt position −200. Since oligonucleotide gvw-3 appears to be specific for *rrnA*, and since gvw-5 hybridized most strongly to DNA fragments corresponding to *rrnA*, most of the transcripts detected by primer extension will probably have arisen from *rrnA*. It is not clear to what extent the RNA 5’ ends detected with gvw-5 could have originated from the second DNA segment to which gvw-5 anneals (Fig. 2).

RNA end-points detected by primer extension at positions −597, −416, −334 and −254 were also detected by nuclease S1 mapping. Furthermore, *in vitro* run-off transcription experiments with *S. coelicolor* RNA polymerase using three different templates revealed that these positions are transcription initiation sites *in vitro*. Together, these data strongly suggest that the RNA end-points at −597, −416, −334 and −254 correspond to the *rrnA* operon.

**Sequence comparison of the *rrnA* promoters**

The promoters P1 and P2 are similar to the consensus sequence for the major class of eubacterial promoters (Hawley & McClure, 1983), with P1 showing a perfect fit to the canonical −35 region. P3 and P4 lack a consensus
Fig. 6. Alignment of the S. coelicolor rRNA promoters. Putative −35 and −10 sequences are underlined and transcription start sites (determined by primer extension) are indicated by dots. The sequence GG(A/G)ATC that occurs in the P2, P3 and P4 −35 and −10 regions is given in bold type. Numbers refer to the sequence shown in Fig. 1(a).

−35 region (Fig. 6). Sequence alignment of P1, P2, P3 and P4 (Fig. 6) shows a striking similarity between the P3 and P4 promoter regions, which are almost identical between positions −1 and −25 with respect to the transcription start site, and have similar −35 regions. These promoters are almost identical to the P3 and P4 promoter of the S. coelicolor rRNA operon (Baylis & Bibb, 1988). Interestingly, the sequence of rRNA A around nt position −35 is comparable to that of rRNA A3 and P4, namely G(G/A)AATC. Boxes with high homology to the sequence G(G/A)AATC can also be seen in the promoter regions of hrdD P2 (GAATTC, ending at −26; the gene encodes a sigma factor homologous to σ^{32B}), abpP2 (GGATTTC, ending at −31; the gene encodes aminoglycoside phosphotransferase) and pA1 (GAACCTC, ending at −31), a promoter which overlaps abpP1, but is oppositely oriented [see Strohl (1992) and references therein].

In addition to the 57 nt direct repeat that encompasses the −35 and −10 regions of P3 and P4, an additional direct repeat of 22 nucleotides is located at nt positions −620 to −599 and −591 to −570 in the region upstream of the 16S rRNA gene (see Fig. 1a), and flanks the P1 transcription start site.

Some clues about recognition of the rRNA A promoters are given by the results of the in vitro transcription assay with the Fsp1–HincII (−708 to −151) fragment as the template (Fig. 5a). We used various RNA polymerase holoenzyme fractions, as used previously for in vitro run-off transcription of the four markedly heterogeneous dagA promoters (Buttner et al., 1987). The rRNA A P1 promoter, which conforms to the consensus sequence for vegetative promoters, is most efficiently transcribed by RNA polymerase present in fraction 28. This is the fraction which is enriched for the RNA polymerase holoenzyme containing σ^{32B}, the vegetative sigma factor, which also transcribes the dagA P4 promoter (M. J. Bibb, unpublished data). This result, in combination with the high homology of P1 with the vegetative promoter consensus sequence, makes it likely that the P1 promoter is recognized by σ^{32B}. For P2–P4, it is less clear which sigma factor is responsible for recognition. Clearly the P3 and P4 promoters are quite similar and the apparent lack of a sequence around the −35 position comparable to the consensus for vegetative promoters suggests that a sigma factor other than σ^{32B} might be responsible for the recognition of these promoters. The P2 promoter is similar to the consensus sequence for the major class of eubacterial promoters, but shares a sequence around its −35 position with P3 and P4.

Relative contribution of the promoters to transcription in vivo

The half-life of rRNA precursors in S. coelicolor is approximately 40 s (Clayton & Bibb, 1990b). In our experiments, mycelial samples were taken at hourly intervals and therefore we can assume that the observed steady-state levels of the primary transcripts represent de novo RNA synthesis. P2 appears to be the most frequently used promoter in fast growing cultures. P1 and P3 are used to similar extents, and are considerably weaker, whereas P4 is the weakest promoter. This contrasts with the results obtained with rRNA D, where P3 and P4 together contributed at least 85% of rRNA D transcription in rapidly growing cultures (Strauch et al., 1991). The discrepancy is surprising since the sequences around P3 and P4 are almost identical for rRNA A and rRNA D.

We believe that nuclease S1 mapping is more reliable for comparing transcript levels than primer extension. In conditions of DNA probe excess, all RNA molecules will be hybridized to the probe and every RNA molecule should contribute to signal strength. This is not always the case for primer-extension experiments, since signal intensity will depend on the ability of the reverse transcriptase to transcribe to the end of the transcript, an event which may occur much less frequently for long RNA molecules than for short ones. In addition, factors such as stem-loop structures in the RNA molecules influence the amount of full-length cDNA made.

Growth-phase-dependent expression of the rRNA operons

Transcription from P1 and P2 in SMM fell markedly between 13 and 17 h after inoculation, which corresponds to the transition between exponential growth and stationary phase. However, transcription from P3 and P4 persists until 17 h after inoculation, and consequently P3 appears to replace P2 as the most frequently used promoter in the transition phase. This observation underlines the need to study relative promoter activities throughout a growth curve.

While the R2-protected fragments are apparent in the early stages of growth, the R1 band did not appear until 14 h after inoculation. This suggests that one of the six rRNA operons may be expressed only late in growth. Such growth-phase-dependent expression might reflect a role for this operon in either morphological differentiation or antibiotic production, and is consistent with the observation by Granovski et al. (1990) of a second phase of stable RNA synthesis on solid medium during the transition from vegetative to aerial mycelium. The intriguing possibility that one of the rRNA operons may be expressed late in growth will be investigated by com-
paring the transcription of all six rrn operons. This should yield information about the relative contribution of the various rrn operons to de novo rRNA synthesis throughout the S. coelicolor life-cycle.

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