**Micromonospora** RNA polymerase activity changes during stationary phase

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RNA polymerase was isolated from *Micromonospora echinospora* and from *Streptomyces lividans*. *In vitro* transcription of a DNA fragment containing multiple tandem promoters from *Micromonospora* followed the pattern of expression observed previously for *in vivo* studies. RNA polymerase was prepared from cultures of *Micromonospora* that were harvested during the growing phase and during the stationary phase. Promoters that were utilized in *Micromonospora* only during the stationary phase were utilized *in vitro* only when RNA polymerase was purified from a stationary-phase culture, and not when RNA polymerase was purified from growing cells.

**Introduction**

*Micromonospora echinospora* (NRRL 15839) is an actinomycete which grows as multicellular mycelia and is capable of forming spores after the growing phase (Maiese et al., 1989). This micro-organism has generated a great deal of interest, due to its production of the calicheamicins (Lee et al., 1989), a novel family of antitumour antibiotics that cause site-specific double-stranded cleavage of DNA (Zein et al., 1988). Because calicheamicin production is temporally regulated, not being made until stationary phase (Baum et al., 1988), we have been particularly interested in promoters of this species that are turned on at this time of the life cycle.

Multiple tandem promoters within a 0·4 kb DNA fragment from *Micromonospora echinospora* have previously been defined. An investigation in which RNA was isolated at different times of the life cycle revealed that three tandem promoters, called Pla, Plb and Plc, were utilized only during stationary phase, while another tandem promoter located downstream, called P2, was utilized during both the growing and stationary phases (Baum et al., 1988). A transformation system has been described for *Micromonospora echinospora*, but problems with plasmid maintenance and/or replication have subsequently proved difficult obstacles to conducting genetic studies in that organism (Love & Rothstein, 1992). Subsequent experiments have relied on the well-developed tools of *S. lividans* for genetic and biochemical studies, hoping that this related actinomycete would recognize the promoters from *Micromonospora*. In fact, all of the *Micromonospora* promoters on the 0·4 kb fragment (P1a, P1b, P1c, and P2) were utilized *in vivo* by *Streptomyces lividans* transformants carrying the 0·4 kb fragment on a plasmid. One additional promoter, Ptk, was utilized by *S. lividans* transformants but not by the native host (Baum et al., 1988, 1989).

There was a close correspondence between the *in vivo* results, and *in vitro* experiments with RNA polymerase isolated from *S. lividans* or *S. coelicolor*, in which runoff transcripts were primed with dinucleotides in order to precisely determine the *in vitro* start sites (Baum et al., 1989). Both the *in vivo* and *in vitro* experiments indicated that the three P1 promoters have unusual structural features. They are very closely spaced, in that P1a, P1b and P1c are each separated by 15 bp, and therefore their recognition sequences probably overlap. A very unusual aspect is the apparent lack of upstream sequence requirements for the P1a and P1b promoters; substituting DNA just 5 bp upstream of P1a and 20 bp upstream of P1b did not drastically alter the utilization of these promoters in *S. lividans* transformants, whereas the same substitutions had a more marked effect on promoter P1c, whose start site is 35 bp from the substitution (Baum et al., 1989).

The one disappointment in the *Streptomyces* experiments was the inability to study the turn-on of expression of the P1 promoters during stationary phase. Unlike *Micromonospora*, *S. lividans* did not exhibit strong temporal regulation of the P1 promoters, even when the
PI promoters were carried on a low copy plasmid (Baum et al., 1988). It was therefore necessary to conduct experiments using the endogenous host to elucidate the mechanism of the temporal regulation of these promoters. In the current study the purification of RNA polymerase from growing and stationary Micromonospora cultures is described, and the *in vitro* RNA polymerase activity as a function of growth phase is examined.

**Methods**

*M. echinospora.* Micromonospora echinospora strain DR46, blocked in the production of calicheamicin (Rothstein & Love, 1991), and *Streptomyces lividans* strain TK54 (Hopwood et al., 1985) were used. Plasmid pEC14 (Baum et al., 1988), containing the 0.4 kb fragment from *Micromonospora* within a *Streptomyces-Escherichia coli* shuttle vector, was the source of templates for the *in vitro* runoff transcription assays.

**Purification of RNA polymerase from Micromonospora and Streptomyces.** Cells were diluted 1/100 into YEME broth (Hopwood et al., 1985) for *Streptomyces* and GER broth (Baum et al., 1988) for *Micromonospora*, and growth at 30 °C was monitored by measuring packed cell volume. Cells were harvested during the growing phase (approximately 20 h incubation) and during stationary phase (45–48 h incubation). RNA polymerase was isolated by Polymun P precipitation, ammonium sulphate precipitation, Sepharose 4B column chromatography, and DNA cellulose chromatography as described by Buttner & Brown (1985), with the following modifications. *Streptomyces* cells were disrupted by treating with lysozyme and sonication (MSE sonicator, 18 micron, 80 pulses of 12 s each on ice) as described by Buttner & Brown (1985). For *Micromonospora* cells, lysozyme treatment was not necessary, and cells were disrupted by sonication. Before DNA cellulose chromatography and after the Sepharose 4B column, RNA polymerase fractions were desalted using Amicon Centriprep 30 units (3 spins at 15000 g for 30 min at 4 °C). This method is easier and considerably faster than dialysis.

**Protein gels.** SDS-PAGE was conducted by the method of Laemmli (1970).

**Transcription studies.** *In vitro* runoff experiments were performed as described by Buttner et al. (1987). Activity was determined using calf thymus DNA (Buttner et al., 1987), and 0.1 units were added to each reaction tube. Each runoff assay was repeated at least three times, and representative experiments are shown in Figs 2 and 3. Templates containing the 0.4 kb region from *Micromonospora* were obtained by digesting plasmid pEC14 as described in Fig. 2(a).

**Results and Discussion**

*Isolation of Micromonospora and Streptomyces RNA polymerase.* To study the temporal regulation of *Micromonospora* promoters, it was necessary to study *Micromonospora echinospora,* rather than *S. lividans* transformants carrying *Micromonospora* DNA, which do not exhibit strong temporal regulation of expression of the PI promoters (Baum et al., 1988). We therefore adapted the methods previously used to purify RNA polymerase from *Streptomyces* to isolate RNA polymerase from *Micromonospora.* We found in particular that lysing by sonication without lysozyme was a more effective way to solubilize RNA polymerase from *Micromonospora* mycelia.

**Composition of Micromonospora RNA polymerase.** The RNA polymerase preparations were subjected to gel electrophoresis (Fig. 1). The β, β', and α subunits of *Micromonospora* enzyme, whether from stationary cells (lane 3) or growing cells (lane 4), were similar in size to the *Streptomyces* subunits (lane 2), rather than those of *E. coli* RNA polymerase (lane 1). This is consistent with the close phylogenetic relationship of *Streptomyces* and *Micromonospora.*

**Comparison of RNA polymerase activities isolated from Streptomyces and Micromonospora.** Previous *in vitro* runoff experiments with *Streptomyces* RNA polymerase and Templates A and B, containing...
Fig. 2. In vitro runoff transcription of Streptomyces and Micromonospora RNA polymerases, using Micromonospora DNA as template. (a) Templates for in vitro transcription experiments were derived by digesting plasmid pEC14 with HindIII and BamHI restriction enzymes (Template A) or by digesting plasmid pEC14 with HindIII and Aul enzymes (Template B). The thick lines indicate Micromonospora DNA from the 0.4 kb fragment containing the promoters Pla, Plb, Plc, Ptk and P2. The transcription products initiating from these promoters are indicated by arrows. The number next to each arrow is the size of the transcript in nucleotides, determined previously (Baum et al., 1989). The size of each template is listed next to the thick line. Restriction sites are Aul (A), BamHI (B), HindIII (H), HincII (Hc), and Sau3Al (S). (b) In vitro runoff transcription products. DNA templates derived from the 0.4 kb fragment of Micromonospora were incubated with the RNA polymerases isolated from Streptomyces or Micromonospora cells in stationary phase, and prepared as described. To each transcription was added 0.1 units of activity, determined by assaying with calf thymus DNA. Lanes: 1, Template A + Streptomyces enzyme; 2, Template A + Micromonospora enzyme; M, molecular mass markers (12P end-labelled HpaII fragments of pBR322 plasmid) with sizes in bp listed; 3, Template B + Streptomyces enzyme; 4, Template B + Micromonospora enzyme. End-to-end transcripts (e) and artificial transcripts (u; see Baum et al., 1989) are indicated.

the 0.4 kb fragment from Micromonospora, established the sizes of transcripts initiating from this region. Furthermore, dinucleotide priming experiments (Fig. 2a) with Streptomyces RNA polymerase and Template B indicated precise start sites for promoters Pla, Plb, Plc, Ptk and P2 (Baum et al., 1989). To compare the transcripts directed by the RNA polymerase preparations from Streptomyces and Micromonospora, the same templates were employed. Streptomyces RNA polymerase isolated from stationary cells synthesized in vitro runoff products, utilizing Template A (Fig. 2b, lane 1), that correspond to the products from the Pl region, from P2, and from Ptk observed previously for this enzyme. The same pattern was observed when Micromonospora RNA polymerase isolated from stationary cells transcribed Template A, except that the Ptk transcript was missing (lane 2). Thus in this respect, the in vitro experiments mirrored transcription in vivo, in that
Streptomyces transformants recognized all of the Micro-
monospora promoters, and the additional promoter, Ptk.
When the shorter Template B was used, the Streptomyces
RNA polymerase transcribed from the P1 promoters and
from P2 and Ptk (lane 3), whereas the Micromonospora
enzyme again transcribed from all the promoters except
for Ptk (lane 4). Thus the in vitro transcription of these
Micromonospora templates agreed with the transcription
observed in vivo, in terms of start sites and species-
specific differences in transcription.

Activity of Micromonospora RNA polymerase isolated
from vegetative and stationary phase cells

The P1 promoters are turned on in vivo in Micromono-
spora during the stationary phase (Baum et al., 1988). To
determine whether a change in RNA polymerase activity might account for this difference in expression, Micromonospora RNA polymerase was prepared from
growing cells (vegetative enzyme) and from stationary
cells (stationary enzyme), and tested for activity using
Templates A and B, shown in Fig. 2(a). The same
amount of activity of vegetative or stationary enzymes
was added to each reaction, using calf thymus DNA as
the standard template.

Fig. 3 shows a comparison of transcription by the
vegetative and stationary enzymes. The vegetative
enzyme synthesized the P2 transcripts of Template A,
but very little of the P1 transcripts (lane 1), whereas the
stationary enzyme synthesized less of the P2 transcripts,
but considerably more of the P1 transcripts (lane 2).
Similarly, when Template B was used, the vegetative
enzyme synthesized the P2 transcripts but almost no P1
transcripts (lane 3), whereas the stationary enzyme
synthesized less of the P2 transcripts and more of the P1
transcripts (lane 4). The change in the transcription
pattern was unambiguous; only the stationary enzyme
had the capacity to transcribe the P1 promoters.

Concluding remarks and future directions

The in vitro experiments showed that the temporal
regulation exhibited in Micromonospora in vivo (Baum et
al., 1988), was reflected as changed in vitro transcriptional activity during stationary phase. Our favoured hypothesis is that the shift in promoter utilization is due
to a new sigma factor(s) present in the stationary RNA
polymerase preparation. This possibility is consistent
with the presence of alternative sigma factors in species
of the actinomycete Streptomyces (Buttner, 1989; Buttner
et al., 1988, 1990; Chater et al., 1989; Westpheling &
Brooner, 1989; Westpheling et al., 1985), in B. subtilis
during sporulation (Losick & Pero, 1981), and in E. coli.

Fig. 3. Temporal changes in RNA polymerase activity isolated from
Micromonospora. RNA polymerase was isolated from Micromonospora
cells harvested during the growing phase (vegetative enzyme), or from
stationary-phase cells (stationary enzyme) as described. These prep-
arations were assayed for runoff transcripts, utilizing Template A and
Template B (Fig. 2a). Each reaction contained 0.1 units RNA
polymerase, determined by assaying with calf thymus DNA as
the standard template. Lanes: 1, Template A + vegetative enzyme; 2, Template
A + stationary enzyme; M, molecular mass markers (32P end-labelled
HpaII fragments of pBR322 plasmid) with sizes in bp listed; 3,
Template B + vegetative enzyme; 4, Template B + stationary enzyme.
End-to-end transcripts (e) and artificial transcripts (u; see Baum et al.,
1989) are indicated.
(Gralla, 1991; Grossman et al., 1984). Conclusive evidence awaits a thorough study of the appearance and effect of subunit proteins on promoter specificity of RNA polymerase, as a function of growth stage in Micromonospora.

To our knowledge, this study is the first biochemical evidence to suggest a change in RNA polymerase promoter selection as a function of growth phase in actinomycetes. It is possible that Micromonospora echinospora confers advantages for studies concerning the temporal regulation of gene expression compared to S. lividans. Consider the case of differentiation. In most Streptomyces species, the development of aerial and substrate mycelia is distinct, suggesting a complex process and a mixed population of cells. Spores, for example, develop only from aerial mycelia which are distinct from substrate mycelia. Sub-populations of cells undergoing distinct developmental paths may well contain different RNA polymerase forms. If a sub-population regulated the promoters occurred during stationary phase in Micromonospora, a clear change in expression of P1 promoters might not be detected if the culture also contained developing, and transforming the calicheamicin producer, Micromonospora RNA polymerase in stationary phase. We thank Bill Maiese for his continued support of the Micromonospora project, and Marcia Osborne for critical reading of the manuscript.

References