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 P1a, P1b and P1c, 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

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P1 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

Strains and plasmids. *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 Polymix 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 P1 promoters (Baum *et al.*, 1988). We therefore adapted the methods

Fig. 1. SDS-PAGE of RNA polymerase preparations from *Streptomyces* and *Micromonospora*. Cells of *Micromonospora* or *Streptomyces* were harvested during the growing phase or the stationary phase. RNA polymerase was isolated as described, subjected to gel electrophoresis [7.5% (w/v) acrylamide, 0.2% bis], and visualized by staining with Coomassie Blue R-250. Lanes: M, molecular mass markers (Bio-Rad); 1, 10 µg RNA polymerase from *E. coli* (Boehringer-Mannheim); 2, 20 µg RNA polymerase from *Streptomyces* stationary-phase cells; 3, 20 µg RNA polymerase from *Micromonospora* stationary-phase cells; 4, 20 µg RNA polymerase from *Micromonospora* growing cells. The origin of the gel samples is indicated by O. Molecular mass markers are labelled to the left, and the α , β , and β' subunits of *E. coli* and *Streptomyces* RNA polymerase are labelled to the right.

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 *Hind*III and *Bam*HI restriction enzymes (Template A) or by digesting plasmid pEC14 with *Hind*III and *Ava*I enzymes (Template B). The thick lines indicate *Micromonospora* DNA from the 0.4 kb fragment containing the promoters P1a, P1b, P1c, 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 *Ava*I (A), *Bam*HI (B), *Hind*III (H), *Hinc*II (Hc), and *Sau*3A1 (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 (32 P end-labelled *Hpa*II 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 P1a, P1b, P1c, 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 P1 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 *Micromonospora* 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 *Micromonospora* 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 & Browner, 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 preparations 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 template. Lanes: 1, Template A + vegetative enzyme; 2, Template A + stationary enzyme; M, molecular mass markers (32 P end-labelled *Hpa*II 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 P1 promoters, so that P1 transcripts were only produced after the growing phase, it might not be detected if the culture also contained another sub-population(s) that expressed the P1 promoters constitutively. In *Micromonospora*, differentiation may be a simpler and more uniform process. In contrast to *S. lividans*, spores can form in liquid media in *Micromonospora*, including GER broth, within approximately seven days of incubation (D. M. Rothstein, unpublished results). The ability of *Micromonospora* to sporulate in liquid culture suggests that the population of cells is differentiating more uniformly. This may account for our observations that a clear change in expression of the P1 promoters occurred during stationary phase in *Micromonospora*, but not in *Streptomyces*.

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