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redD and *act*II-ORF4, Pathway-Specific Regulatory Genes for Antibiotic Production in *Streptomyces coelicolor* A3(2), Are Transcribed In Vitro by an RNA Polymerase Holoenzyme Containing σ^{hrdD}

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***redD* and *act*II-ORF4, regulatory genes required for synthesis of the antibiotics undecylprodigiosin and actinorhodin by *Streptomyces coelicolor* A3(2), were transcribed in vitro by an RNA polymerase holoenzyme containing σ^{hrdD}. Disruption of *hrdD* had no effect on antibiotic production, indicating that *redD* and *act*II-ORF4 are transcribed in vivo by at least one other RNA polymerase holoenzyme. These data provide the first experimental evidence that HrdD can function as a σ factor.**

Although at least eight different σ factors have been identified in *Streptomyces coelicolor* A3(2) (reference 3 and references therein, 8, 17a, 19), the functions of several remain to be determined. The principal and essential σ factor, σ^{hrdB} (2, 4), belongs to a family of four extremely similar proteins that includes putative σ factors σ^{hrdA}, σ^{hrdC}, and σ^{hrdD} (24). Several studies have revealed that regions 2.4 and 4.2 of σ factors are responsible for recognizing the −10 and −35 hexamers, respectively, of cognate promoters (13). Each of the amino acids at the three positions in region 4.2 thought to be directly involved in recognition of the −35 region is identical in HrdA to D (3, 17), and the amino acid sequences of region 2.4 of HrdA to D show high levels of identity and similarity (3, 17). It is thus probable that HrdA, HrdC, and HrdD recognize promoters that differ only slightly, or not at all, from the proposed consensus sequences for σ^{hrdB} (TTGACN-16 to 18 bp-TAGAPuT) (22).

In liquid-grown cultures of *S. coelicolor*, production of the pigmented antibiotics undecylprodigiosin and actinorhodin usually occurs in stationary phase, after transcriptional activation of the pathway-specific regulatory genes *redD* (23) and *act*II-ORF4 (12), respectively. Given the role that alternative σ factors play in growth phase-dependent gene expression in other bacteria (1, 9, 14, 16), we set out to establish which form of RNA polymerase holoenzyme transcribes *redD* and *act*II-ORF4.

In vitro transcription of *redD* and *act*II-ORF4. Earlier studies of liquid (SMM [23])-grown cultures of *S. coelicolor* A3(2) strain M145 had revealed maximal levels of *act*II-ORF4 and *redD* transcripts during the transition phase (12 and 23, respectively). Consequently, RNA polymerase was isolated from a 4-liter transition phase culture of M145 that had been grown in SMM (23) and that had just begun to make undecylprodigiosin, and the different holoenzymes were partially separated by Superose 6 fast protein liquid chromatography as described by Buttner et al. (7). The fractions obtained were used in in vitro runoff transcription assays with DNA templates containing either the *dagA* promoter region, which contains four different promoters ($dagAp_1$ to p_4) transcribed by at least three, and probably four, different RNA polymerase holoenzymes (7), or the *redD* promoter region, which contains, in addition to *redDp*, four divergently oriented promoters, Pr1 to Pr4 (18, 23; Pr1 to Pr4 read into an open reading frame whose product is homologous to TrkA of *Escherichia coli*, a protein involved in potassium transport [20a]). While transcription from $dagAp_4$, which is recognized by the RNA polymerase holoenzyme containing essential σ factor σ^{hrdB} (2, 4), was greatest in fraction 1, *redDp* transcription was most active in fraction 2 (Fig. 1). A profile essentially identical to that observed for *redDp* was obtained with a DNA template containing *act*II-ORF4*p* (data not shown). These results suggested that both *redD* and *act*II-ORF4 might be transcribed in vitro by an RNA polymerase holoenzyme that contains a σ factor other than σ^{hrdB}.

Reconstitution experiments. RNA polymerase fractions 1 to 4 were pooled, and a sample was subjected to preparative-scale sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Coomassie brilliant blue R-stained bands between 66 and 29 kDa were excised from the gel, and the proteins contained in each gel slice were eluted and renatured in the presence of GroEL (2). Samples of the renatured proteins were added to *E. coli* core RNA polymerase (Epicentre Technologies, Madison, Wis.) as described in reference 2, and their ability to stimulate in vitro runoff transcription from *redDp* was assessed. Reconstitution activity was greatest with gel slice 6 (Fig. 2) (which also contained the α subunit of RNA polymerase with an apparent molecular size of 45 kDa); a low level of in vitro transcription was also observed with gel slice 1, which contained σ^{hrdB}. Essentially identical results were obtained with *S. coelicolor* core RNA polymerase (prepared as described in reference 7). The proteins contained in gel slice 6 were subjected to two-dimensional PAGE (20), which revealed a major spot and a minor species that migrated on SDS-PAGE with an apparent molecular size of 46 kDa. Both spots were blotted onto a polyvinylidene difluoride membrane, and their N-terminal amino acid sequences were determined. The major spot corresponded to the α subunit (3a), and the first eight amino acid residues of the minor protein component (XAX RAVAR, where X is unassigned) corresponded to those of

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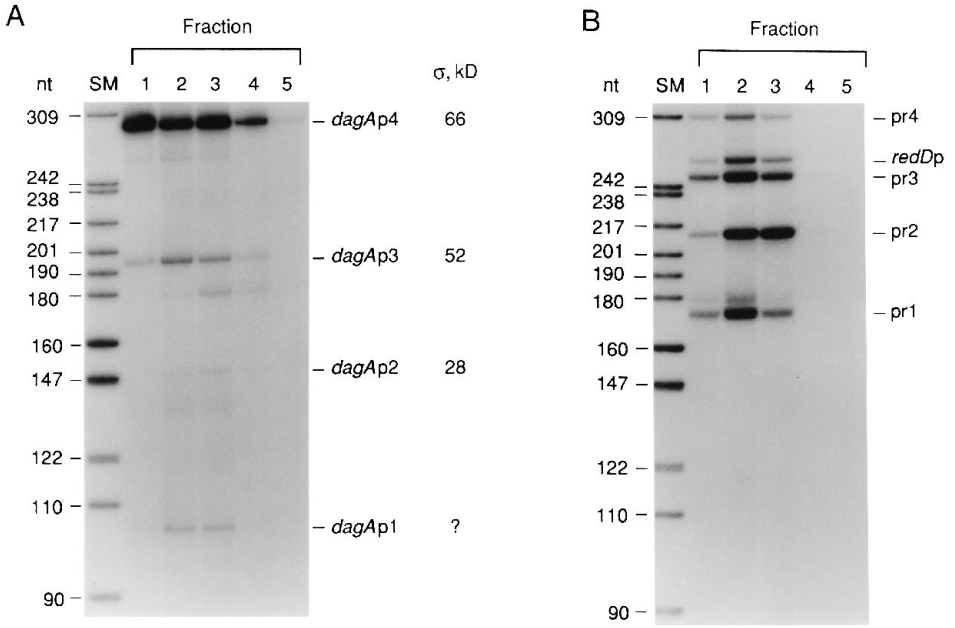

FIG. 1. In vitro runoff transcription assays of fractions obtained after Superose 6 fast protein liquid chromatography of total RNA polymerase isolated from a transition phase culture of *S. coelicolor* M145. (A) In vitro runoff transcripts obtained with a 589-bp *Sma*I-*Ava*II fragment that should give runoff transcripts for *dagAp*1 to 4 of 99, 145, 191, and 285 nucleotides (nt), respectively (5). The apparent size of the σ factor responsible for initiation of transcription at each promoter is shown to the right (7). (B) In vitro runoff transcripts obtained with a 762-bp *Nae*I fragment containing *redDp* and divergently oriented Pr1 to Pr4 (18, 23), which should give runoff transcripts of 265 nucleotides for *redDp* and 177, 213, 247, and 306 nucleotides for P1 to P4, respectively (18, 23). SM, end-labelled, *Hpa*II-digested pBR322 size markers. Reactions were carried out under conditions of DNA template limitation. kD, kilodaltons.

σ^{hrdD} (fMATRAVAR) (24). The nucleotide sequence of *hrdD* predicts a product of 37.2 kDa. σ factors frequently migrate more slowly than expected on SDS-PAGE, a characteristic attributable to their unusually high negative charge (17a, 21).

To assess whether the minor protein component alone could reconstitute transcription from *redDp* and *act*II-ORF4*p* in vitro, a further sample of RNA polymerase (fractions 1 to 4 of Fig. 1) was subjected to preparative-scale SDS-PAGE under conditions that resolved the minor protein from the α subunit (data not shown). The minor protein band was eluted, this time from gel slice 8, renatured, and used in in vitro runoff transcription assays with samples of *S. coelicolor* and *E. coli* core RNA polymerases. The 46-kDa protein conferred on both core enzymes the ability to initiate transcription at *redDp* and *act*II-ORF4*p* (Fig. 3); material eluted from gel slices 7 and 9 had no stimulatory effect. The 46-kDa protein also elicited transcription from *dagAp*4 with the *E. coli* core enzyme but not with that from *S. coelicolor* (Fig. 3).

Disruption of *hrdD*. To determine whether the 46-kDa protein was σ^{hrdD}, a *hrdD* mutant of *S. coelicolor* M145 was constructed by using KC926, an *attP* derivative of the temperate actinophage ϕC31 which contains a segment of DNA internal to the *hrdD* coding region (4). KC926 and KC301 (an $attP^+$ control phage) (15) were used to lysogenize *S. coelicolor* M145 by selecting for thiostrepton resistance (one of the antibiotic resistance markers carried by both phages) as described by Buttner et al. (4). KC926 can give rise to lysogens only by homologous recombination between the cloned segment and the chromosomal copy of *hrdD*; the resulting disruption of *hrdD* was subsequently confirmed by Southern analysis of *Sma*I-cleaved M145 and M145(KC926) DNAs by using the 1.3-kb *Eco*RI-*Kpn*I fragment containing *hrdD* as a ^{32}P-labelled hybridization probe (4). The phenotypes of M145(KC301) and M145(KC926) when grown in SMM and on a solidified version containing 15 g of agar per liter (but lacking antifoam and PEG 6000) were identical, indicating that *hrdD* is not essential for the production of either undecylprodigiosin or actinorhodin. The same conclusions were drawn by Buttner et al. (4), who

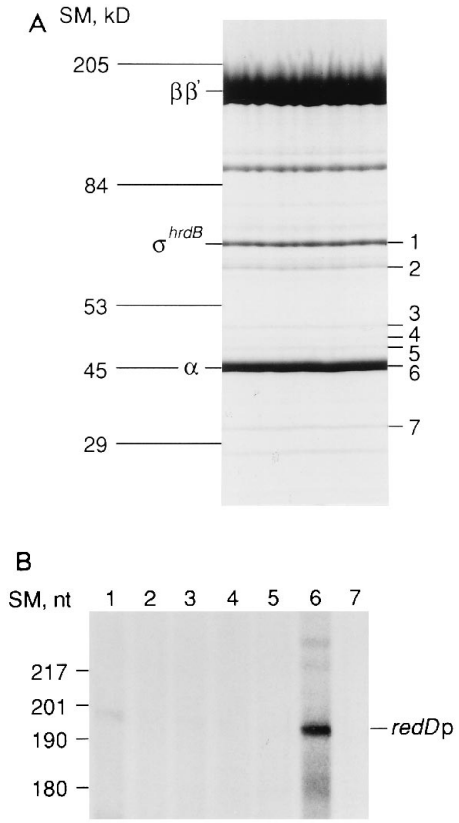

FIG. 2. (A) Coomassie brilliant blue R-stained SDS-PAGE of pooled fractions 1 to 4 of Fig. 1. SM, size markers; kD, kilodaltons. (B) The protein bands marked 1 to 7 in panel A were excised, eluted, renatured in the presence of GroEL, and used in in vitro runoff transcription reconstitution assays with *E. coli* core RNA polymerase and a DNA template containing *redDp* (the 349-bp *Bam*HI fragment from pIJ4097) (1), which is a cloned PCR product containing only *redDp* and should give a runoff transcript of 198 nucleotides (nt). SM, end-labelled, *Hpa*II-digested pBR322 size markers.

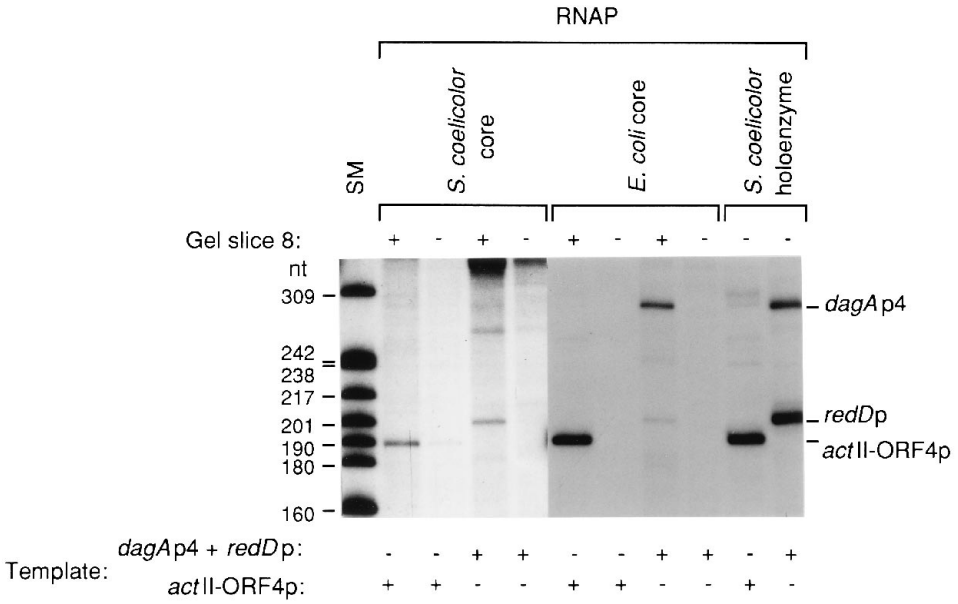

FIG. 3. In vitro runoff transcription reconstitution assays using the renatured 46-kDa protein (gel slice 8) and *S. coelicolor* and *E. coli* core RNA polymerases. The positions expected for runoff transcripts from *dagAp*4, *redDp*, and *act*II-ORF4*p* are shown to the right. The DNA templates used for *dagAp*4 and *redDp* are described in the legends to Fig. 1 and 2, respectively; for *act*II-ORF4*p*, a 371-bp *Ava*II fragment was used (11), which should give a runoff transcript of 187 nucleotides (nt) (10). SM, end-labelled, *Hpa*II-digested pBR322 size markers.

used *hrdD* mutants of two different *S. coelicolor* strains, J1501 and J1507, and different growth conditions. RNA polymerase was isolated from YEME (15)-grown transition phase cultures of M145 and M145(KC926); analysis of each preparation by two-dimensional PAGE showed that the 46-kDa protein was absent from the *hrdD* mutant (Fig. 4). Both preparations were subjected to preparative-scale SDS-PAGE, gel slices containing and flanking the α subunit were excised, and the proteins contained therein were eluted, renatured with GroEL, and used in in vitro runoff transcription assays with *E. coli* core RNA polymerase. The fraction from M145 containing the α subunit, and presumably HrdD, conferred the ability to initiate transcription at *redDp*, but none of the fractions from the *hrdD* mutant strain did so (Fig. 5). Moreover, when samples of each RNA polymerase preparation were used in in vitro runoff transcription assays with DNA templates containing *dagAp*4 and *redDp*, the ability of the RNA polymerase isolated from the *hrdD* mutant to initiate transcription at *redDp* was severely reduced, while the levels of *dagAp*4 transcribing activity of both appeared to be the same (data not shown).

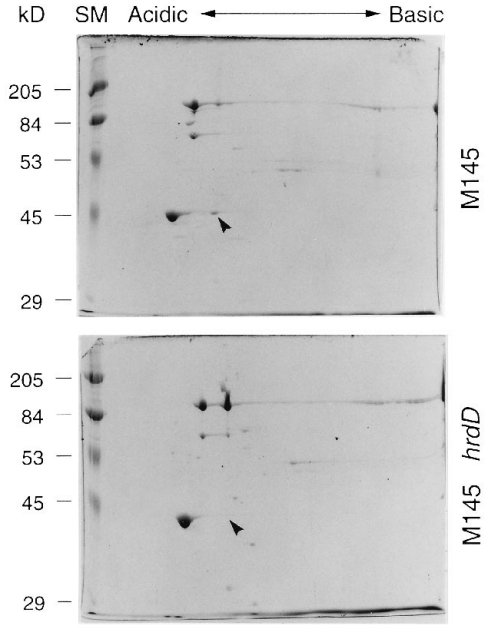

FIG. 4. Two-dimensional PAGE of RNA polymerase isolated from YEME-grown transition phase cultures of M145 and M145 *hrdD*. The position of the 46-kDa HrdD protein, which is absent from M145 *hrdD*, is indicated by the arrowhead. kD, kilodaltons. SM, size markers.

Concluding remarks. While *redDp* and *act*II-ORF4*p* are recognized almost exclusively in vitro by an RNA polymerase holoenzyme containing σ^{hrdD}, disruption of *hrdD* had no effect on the production of either undecylprodigiosin or actinorhodin. Consequently, both promoters must be recognized in vivo by at least one other σ factor. In earlier studies, *hrdA*, *hrdC*, and *hrdD* were all inactivated in a triple mutant without any obvious effect on phenotype (6), suggesting that neither *redD* nor *act*II-ORF4 is solely dependent on any of the minor *hrd* genes for its transcription. Since we detected a low level of in vitro transcription from *redDp* with the core enzyme and a protein corresponding in size to σ^{hrdB} (Fig. 2), we surmise that σ^{hrdB}, perhaps with the assistance of a positively acting regulatory protein that is absent from the RNA polymerase preparation, recognizes both *redDp* and *act*II-ORF4*p* in vivo; this is consistent with the potential −10 and −35 sequences of *redDp* (23) and *act*II-ORF4*p* (10), each of which is similar to the proposed consensus sequence for the major holoenzyme (22). While it is possible that the ability of σ^{hrdD} to recognize *redDp* and *act*II-ORF4*p* in vitro is an artifact of the assay conditions used and that the σ factor plays no role in the transcription of either *redD* or *act*II-ORF4 in vivo, our data establish for the first time that HrdD has σ factor activity. Several promoters from *E. coli* are recognized by more than one σ factor in vitro. For instance,

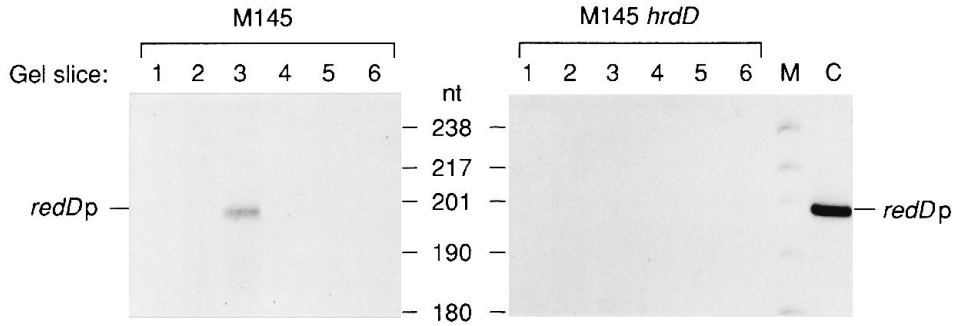

FIG. 5. In vitro runoff transcription reconstitution assays using SDS-PAGE-fractionated RNA polymerase isolated from M145 (left panel) and M145 *hrdD* (right panel) with *E. coli* core RNA polymerase. The α subunit of RNA polymerase and the 46-kDa HrdD protein (in M145 only) occur in gel slice 3. The DNA template used is described in the legend to Fig. 2 and should give a runoff transcript for *redDp* of 198 nucleotides (nt). C, in vitro runoff transcripts obtained with a sample of pooled fractions 1 to 4 of Fig. 1. M, end-labelled, *Hpa*II-digested pBR322 size markers.

σ^{70} promoters *lacUV5p*, *trpp*, and *dnaQp*2 are also recognized in vitro by σ^S (25), while transcription of σ^S-dependent promoters *bolAp*1 and *xthAp* is also directed in vitro by σ^{70} (18a). Although the in vivo significance of such overlapping specificities is unclear, it is possible that *redDp* and *act*II-ORF4*p* are recognized in vivo by both σ^{hrdD} and σ^{hrdB}, an apparent redundancy that might reflect substantial changes in the relative activities of the two σ factors during growth and development.

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