Transcription Apparatus and Its Relatives in *Streptomyces*

Hiddenori Shinkawa

Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Kagamiyama 1-4-1, Higashi-Hiroshima 739, Japan

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Transcription is the first event to realize the biological functions. Initiation of transcription is the association of RNA polymerase with the *cis*-acting DNA sequence which is termed promoter. RNA polymerase holoenzyme of bacteria consists of five subunits ($\alpha_2\beta\beta'\sigma$)¹⁾. Sigma (σ) subunit is required for the promoter recognition, while core enzyme ($\alpha_2\beta\beta'=E$) is able to catalyze RNA synthesis. Most prokaryotic genes are regulated at the level of transcription. This is economical for bacteria and enables to switch the gene expression dramatically. Transcriptional regulation plays a critical role in prokaryotic differentiation. From studies on the sporulation in *Bacillus subtilis*, it is established that substitution of σ subunit changes the promoter recognition of RNA polymerase and then induces the expression of a particular set of genes at a particular stage of cell cycle²⁾. Sigma substitution also enables the tight regulation of particular promoters in other bacteria for the special functions such as heat shock response, flagella synthesis and nitrogen fixation¹⁾.

Streptomycetes are highly differentiating bacteria. These organisms are also notable for the production of various secondary metabolites, including commercially important antibiotics. The accumulation of knowledge on the transcriptional control in *Streptomyces* is the most important to understand the regulation of differentiation and secondary metabolite production, but it is not enough yet. Moreover, such understandings will be useful after completing the genome project of *Streptomyces*. Further information is required. In addition,

streptomycete genome has high G+C contents (more than 70%). The feature of *Streptomyces* promoter is of special interest, since *Escherichia coli* promoter has A+T rich sequence to facilitate the melting of double strand DNA³⁾.

Promoter diversity in *Streptomyces*

To date, a number of genes have been isolated from *Streptomyces*. Promoter analyses indicated a large diversity in the sequence^{4,5)}. The author also tried to classify, but it was difficult to classify the *Streptomyces* promoters by nucleotide sequence. This unusual feature of *Streptomyces* promoters may reflect the high G+C content of genome and the RNA polymerase heterogeneity. There are some problems on the characterization of *Streptomyces* promoter. The first, 5' end of mRNA was usually determined by S1 nuclease mapping or primer extension experiment, but it does not always represent a transcription start point (tsp). It is one of the processed end from large mRNA at times. The second, the cloned genes are biased due to the particular interests in antibiotics production. The data for indispensable genes are insufficient. The third, most works on *Streptomyces* promoter were according to the analogy from the results of *E. coli* and *B. subtilis* promoters. Some of *Streptomyces* promoters might be out of the common knowledge of *E. coli* and *B. subtilis*. For the reasons described above, it is hard to establish the consensus sequence of *Streptomyces* promoter only from nucleotide sequence. Interaction of RNA polymerase with promoter, namely protein-DNA interaction,

should be analyzed. I then started to examine the correlation between RNA polymerase heterogeneity and promoter sequence variety.

For analysis of *Streptomyces* promoters, especially indispensable genes, several genes were cloned from *S. griseus*: ribosomal RNA genes (*rrnA*, *B*, *C*, *D*, *D'*, *E*)⁶, *dnaK* operon (*dnaK*, *grpE*, *dnaJ*, *orfX*)⁷, genes for principal σ factor of RNA polymerase (*hrdA*, *B*, *D*)^{8,9}, α operon (*map*, *infA*, *rpmJ*, *rpsM*, *rpsK*, *rpoA*, *rplQ*, *hisT*)¹⁰, β operon (*rpoB*, *C*)¹⁰, S2 operon (*rpsB*, *tsf*)¹⁰, and part of *str* gene cluster for streptomycin biosynthesis (*strD*, *strR*, *aphD*, *strB*, *strF*, *strG*)¹¹. The DNA sequence and the *tsp* of these promoters were determined, and the expression properties were analyzed by using the promoter fusion with *mel* gene. In addition, nine promoters were isolated from *S. griseus* genome by shotgun cloning method using a promoter-probe vector pML104⁸ and characterized as well as the above genes. In brief, they showed various modes on the time and the strength of expression, and some of them affected the development of morphology or the growth rate. Interestingly, significant differences were observed in some of them on the expression properties between in liquid culture and on solid medium.

Property of RNA polymerase from vegetative mycelia of *S. griseus*

Recently, the author has established the purification of RNA polymerase from the exponentially growing mycelia of *S. griseus* by two steps of ion-exchange chromatography (DEAE-Toyopearl and phosphocellulose)¹². The author also tried DNA-cellulose column chromatography, but the phosphocellulose column chromatography was more effective for separation of the *Streptomyces* enzyme. The phosphocellulose column purification system is also greater than DNA-cellulose system on the reproduction. The subunits of core enzyme (α , β , β') were found to have apparent molecular weights of

49, 140, and 150 kDa, respectively. The 45 kDa band may correspond to the Y-band which was found to associate with the purified *S. antibioticus* enzyme¹³, although its function is unclear. Significant amounts of σ^{hrdB} with a molecular weight of 66 kDa was dissociated from the core enzyme during fractionation through a DEAE column. The *S. griseus* σ^{hrdB} protein seems to associate loosely with the core enzyme. This might facilitate the binding of alternative σ factors to the core enzyme. Using the purified holoenzyme, *in vitro* transcription system was established. $E\sigma^{\text{hrdB}}$ of *S. griseus* was found to transcribe not only the *dagAp4* promoter but also some of the essential genes (*hrdB* and *dnaK*) of *S. griseus* *in vitro* (Fig. 1). *In vitro* transcription system is a powerful tool for

Fig. 1. *In vitro* transcription with $E\sigma^{\text{hrdB}}$ holoenzyme. Template DNA, shown on top of each lane, was incubated with the holoenzyme ($E\sigma^{\text{hrdB}}$) and the single-round transcription was then carried out by the addition of substrate-heparin mixture. The specific transcripts from each promoter are shown by arrowheads.

Table 1. σ factors and putative transcription factors

Factor	Recognized promoter	Function	Evidence ^{a)}	Reference
σ^{28} (SigE)	<i>dagA</i> p2	unknown	B, G	15, 18
σ^{35}	<i>veg</i>	unknown	B	14
σ^{49} (σ^{52})	<i>ctc</i> , <i>dagA</i> p3	unknown	B	14, 15
σ^{66} (HrdB)	<i>veg</i> , <i>dagA</i> p4, <i>XP55</i> , <i>dnaK</i> , <i>hrdB</i>	essential	B, G	12, 15, 23
HrdA	unknown	unknown	G	9, 18, 19, 20
HrdC	unknown	unknown	G	18, 19
HrdD	<i>redD</i> , <i>actII</i> -ORF4	unclear	B, G	9, 18, 19, 20, 29
HrdE	unknown	unknown	G	20
SigF	unknown	sporulation	G	26
WhiG	<i>B. subtilis</i> σ^D -dependent type?	sporulation	G	25
WhiB	unknown	sporulation	G	27

a) B and G represent biochemical and genetical evidences, respectively.

transcriptional analysis.

In addition, the *dnaK* gene was transcribed with a major form of RNA polymerase holoenzyme ($E\sigma^{hrdB}$) from single promoter (*tsp*) which was induced by heat shock. It suggests that the mechanism of heat shock response in *Streptomyces* is different from that in *E. coli*.

Heterogeneity of RNA polymerase in *Streptomyces*

tomyces

To date, the identified σ species and the putative σ -like transcription factors from *Streptomyces* sp. are listed in Table 1. In *S. coelicolor* A3(2), five different forms of σ factors, σ^{28} , σ^{35} , σ^{49} , σ^{52} (probably equivalent to σ^{49}) and σ^{66} , have been identified; they associate with the core enzyme and determine the specificity of promoter recognition^{14,15}.

<i>hrdBp</i>	GGATTGGCCGTAACACTCCTCGAAGCAGCGGATGACTTAAGAG
	* * * * *
<i>dnaKp</i>	CAGGTCTGTTGACTCCGGCGTCGGCGTCATGCATTCTTGAGTC
	* * * * *
<i>dagAp4</i>	TACCGATTGTCAACCCTGCGACACTCCGCTGTAGCATTTCGGGAAA
	*** ** * * *
<i>veg</i>	TTTATTTGACAAAAATGGGCTCG TGTTGTACAATAAATGTA
σ^{70} consensus	TTGACA TATAAT
	-35 -10

Fig. 2. Alignment of the promoters transcribed with $E\sigma^{hrdB}$. The promoters *hrdBp* and *dnaKp* of *S. griseus*, and *dagAp4* of *S. coelicolor* were transcribed with $E\sigma^{hrdB}$. Brown *et al.* showed $E\sigma^{hrdB}$ transcribed *veg* promoter²³. Shaded nucleotide residues are conserved among four promoters described above. Transcription start point is underlined.

On the other hand, Southern hybridization analysis using a σ -specific probe revealed the presence of at least four genes which encode putative σ factors with high similarity to the principal σ factors, σ^{70} and σ^A , of *E. coli* and *B. subtilis*, respectively¹⁶. These genes, named *hrdA*, *hrdB*, *hrdC*, and *hrdD* (homologs of *rpoD*), have been isolated from *S. coelicolor*¹⁷⁻¹⁹. Similar genes were cloned from *S. aureofaciens*²⁰ and *S. griseus*^{8,9}, but *hrdC* is missing in both strains. *S. aureofaciens* has an extra *hrd* gene (*hrdE*) which is highly similar to *hrdB*. It is of interest in which stage of cell cycle these *hrd* genes are involved. The *hrdB* gene probably exists in all *Streptomyces* strains^{9,21}, and its gene product (HrdB) may be essential for cell growth based on the gene disruption experiments¹⁹. Therefore, HrdB might be involved in the transcription of indispensable genes. HrdB

in *Streptomyces* could be a counterpart of σ^{70} in *E. coli* or σ^A in *B. subtilis*. The *hrdB* gene is known to encode σ^{66} species²³. It directs transcription from the *S. coelicolor* *dagA* p4 and *B. subtilis* *veg* promoters, both carrying a DNA sequence similar to the *E. coli* consensus promoter sequence¹⁵. HrdB of *S. griseus* is also 66 kDa polypeptide¹². I found that $E\sigma^{hrdB}$ holoenzyme could transcribe the *hrdB* promoter and the *dnaK* promoter *in vitro*, which were included in indispensable genes¹². These sequence were not so similar to *dagA*p4 promoter and *veg* promoter (Fig. 2). Furthermore, *lacUV5* promoter, typical *E. coli* promoter³, was not transcribed *in vitro* with $E\sigma^{hrdB}$ of *S. griseus*, whereas it could express in *Streptomyces* cells and was effectively transcribed *in vitro* with $E\sigma^{70}$ of *E. coli*. The important signals for transcription such as -10 and -35 signals should be

Fig. 3. The nucleotide sequence of oligoprobes deduced from the N-terminal amino acid sequence of core RNA polymerase from *S. griseus*.

determined carefully.

The functions of other Hrd proteins are still unclear. The disruption mutants of *hrdA*, *hrdC* and *hrdD* had no obvious phenotype on growth, morphology, and antibiotic production in *S. coelicolor* A3(2)^{19,22}. However, *hrdA* and *hrdD* must have the significant functions, since they

are distributed in *Streptomyces* strains^{9,21}) and probably other actinomycetes as widely as *hrdB*. For example, it is expected that one or both of them is/are the same species of *E. coli* σ^S which is involved in the physiological homeostasis in stationary phase²⁴). In addition, the genes, *whiG* and *sigF* may encode alterna-

Fig. 4. Alignment of three Hrd products of *S. griseus*. Shaded amino acid residues indicate the conserved residues among three Hrd proteins. The subregions 1.2 through 4.2 which possess the functionally important domains are shown by dotted line. Thick overlines represent the domain for core binding and the 'rpoD box' conserved among primary σ factors. Vertical arrowheads indicate the amino acid residues which are thought to contact with the -10 signal. Thick underlines represent the possible helix-turn-helix motif involved in -35 recognition.

tive σ factors from the results of nucleotide sequence^{25,26}, and the *whiB* product resembles to the transcription factors of eukaryotes²⁷. These genes are involved in the morphological development of *S. coelicolor* A3(2) and *S. aureofaciens*.

No promoter recognized with these gene products except HrdB (σ^{66}) and SigE (σ^{28})^{15,28} was identified by *in vitro* transcription experiment so far. Most recently, it was shown that HrdD with an apparent molecular weight of 45 kDa could direct the transcription from the promoters of *redD* and *actII-ORF4* which were also transcription factors required for the activation of antibiotic-biosynthesis pathway, whereas disruption of the *hrdD* gene had no effect on antibiotic production²⁹. Studies on the products of putative σ factor genes like the HrdD are necessary to elucidate their functions.

Cloning of the genes for RNA polymerase

To construct the recombinant RNA polymerase of *Streptomyces*, the genes for each subunit

have been cloned. RNA polymerase of *S. griseus* consists of four distinct subunits (α , β , β' , σ). Each N-terminal sequence of the subunits of *S. griseus* core enzyme was determined by the Edman degradation method (Fig. 3). The oligonucleotides were designed from the results of amino acid sequence on the basis of the biased codon usage of *Streptomyces*³⁰. Then, the genes for each subunit of core enzyme were isolated from the cosmid library of *S. griseus*³¹. A gene for Y-band was also isolated in the same manner.

The α operon consisted of a gene for α subunit (*rpoA*) and several genes for translation. The genes of α operon were arranged into the following order; *map-infA-rpmJ-rpsM-rpsK-rpoA-rplQ-hisT*. They were probably transcribed into a large mRNA. The genes for β and β' subunits (*rpoB* and *rpoC*, respectively) were arranged into *rpoB-rpoC*. DNA sequence also revealed that a gene for Y-band encoded *rpsB* for ribosomal protein S2. It is of great interest whether the transcription apparatus communi-

Fig. 5. Hypothetical model of Hrd- σ factor equilibrium in *Streptomyces*. Some of indispensable genes recognized only by HrdB- σ factor. Some of streptomycete promoters are transcribed with two or more holoenzymes.

cates with translation apparatus.

The alternative σ factor genes such as *whiG*, *sigE* and *sigF* have already isolated from *S. griseus*. Analyses of them are in progress. On the other hand, three genes for principal σ factor (*hrdA*, *hrdB*, *hrdD*) were isolated from *S. griseus*. Alignment of their products is shown in Fig. 4. Significant homology was observed in the region 2. Similarity was also observed in the region 4. The region 2 is divided into four subregions (2.1 through 2.4). The region 4 is also divided into 4.1 and 4.2. These subregions seem to play the important roles, such as core binding, -10 recognition, and -35 recognition from the results of other bacteria. The subregion 2.4 is thought to bind to the -10 signal of promoter, and the subregion 4.2 is thought to be involved in -35 recognition³²). If so, Hrd proteins should share some of promoters each other, because of the homology in 2.4 or 4.2. The hypothetical model is shown in Fig. 5. It is necessary to identify the promoters which depend on the Hrd σ factors, and to analyze the interactions of purified Hrd proteins with the promoters.

More and more materials are prepared to analyze the transcription in *Streptomyces*. The recombinant RNA polymerase is now ready to be constructed. The reconstitution experiments of Hrd proteins and other alternative σ factors with the core enzyme are in progress to understand their functions.

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REFERENCES

- 1) Ishihama, A.: Protein-protein communication within the transcription apparatus. *J. Bacteriol.* 175: 2483-2489, 1993.
- 2) Losick, R.; P. Youngman & P.J. Piggot: Genetics of endospore formation in *Bacillus subtilis*. *Annu. Rev. Genet.* 20: 625-669, 1986.
- 3) Ishihama, A.: Promoter selectivity of prokaryotic RNA polymerases. *Trends Genet.* 4: 282-286, 1988.
- 4) Strohl, W. R.: Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucl. Acid. Res.* 20: 961-974, 1992.
- 5) Bourn, W. R. & B. Babb: Computer assisted identification and classification of streptomycete promoter. *Nucl. Acids Res.* 23: 3696-3703, 1995.
- 6) Sahara, H.; K. Inada, H. Shinkawa, H. Kinashi & O. Nimi: Promoter analysis of the rRNA operons of *Streptomyces griseus*. *Nippon Nogei-kagaku Kaishi* (Japanese) 69: 7, 1995.
- 7) Hatada, Y.; H. Shinkawa, K. Kawamoto, H. Kinashi & O. Nimi: Cloning and nucleotide sequence of a *hsp70* gene from *Streptomyces griseus*. *J. Ferment. Bioeng.* 77: 461-467, 1994.
- 8) Shinkawa, H.; Y. Hatada, M. Okada, H. Kinashi & O. Nimi: Nucleotide sequence of a principal sigma factor gene (*hrdB*) of *Streptomyces griseus*. *J. Biochem.* 118: 494-499, 1995.
- 9) Shinkawa, H.; K. Kawamoto, Y. Ohmori, H. Kinashi & O. Nimi: Cloning and nucleotide sequence of the genes for *hrdA* and *hrdD*: members of *rpoD* family in *Streptomyces griseus*. *Actinomycetol.* 118: 494-499, 1996.
- 10) Shinkawa, H.; Y. Mori; H.W. Luo; T. Hasegawa & O. Nimi: Transcriptional analysis of the genes for RNA polymerase core enzyme of *Strepto-*

- myces griseus*. In "Abstract from 19th Annual Meeting of The Molecular Biology Society of Japan" pp.447, 1996.
- 11) Shinkawa, H.; M. Sugiyama & O. Nimi: Two genes for streptomycin phosphotransferase in *Streptomyces griseus*. In "Biology of Actinomycetes '88" (ed. Y. Okami, T. Beppu, & H. Ogawara), pp.353-358, Japan Scientific Societies Press, Tokyo, 1988.
 - 12) Shinkawa, H.; N. Fujita, T. Shiina, K. Tanaka, H. Takahashi, A. Ishihama & O. Nimi: Purification and characterization of RNA polymerase from vegetative-phase mycelia of *Streptomyces griseus*. J. Biochem. 118: 488-493, 1995.
 - 13) Jones, G.H.: Purification of RNA polymerase from actinomycin producing and nonproducing cells of *Streptomyces antibioticus*. Arch. Biochem. Biophys. 198: 195-204, 1979.
 - 14) Westpheling, J.; M. Ranes & R. Losick: RNA polymerase heterogeneity in *Streptomyces coelicolor*. Nature. 313: 22-27, 1985.
 - 15) Buttner, M.J.; A.M. Smith & M.J. Bibb: At least three different RNA polymerase holoenzymes direct transcription of the agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2). Cell. 52: 599-607, 1988.
 - 16) Tanaka, K.; T. Shiina & H. Takahashi: Multiple principal sigma factor homologs in eubacteria: identification of 'rpoD box'. Science. 242: 1040-1042, 1988.
 - 17) Shiina, T.; K. Tanaka, & H. Takahashi: Sequence of *hrdB*, an essential gene encoding sigma-like transcription factor of *Streptomyces coelicolor* A3(2): homology to principal sigma factors. Gene 107: 145-148, 1991.
 - 18) Tanaka, K.; T. Shiina & H. Takahashi: Nucleotide sequence of genes *hrdA*, *hrdC* and *hrdD* from *Streptomyces coelicolor* A3(2) having similarity to *rpoD* gene. Mol. Gen. Genet. 229: 334-340, 1991.
 - 19) Buttner, M.J.; K.F. Chater & M.J. Bibb: Cloning, disruption, and transcriptional analysis of three RNA polymerase sigma factor genes of *Streptomyces coelicolor* A3(2). J. Bacteriol. 172: 3367-3378, 1990.
 - 20) Kormanec, J.; M. Farkašovsky & L. Potůčková: Four genes in *Streptomyces aureofaciens* containing a domain characteristic of principal sigma factors. Gene. 122: 63-70, 1992.
 - 21) Takahashi, H.; K. Tanaka & T. Shiina: Genetic constituent of *rpoD* gene homologs in *Streptomyces* strains. In "Biology of Actinomycetes '88" (ed. Y. Okami, T. Beppu, & H. Ogawara) pp.58-63, Japan Scientific Societies Press, Tokyo, 1988.
 - 22) Buttner, M.J. & C.G. Lewis: Construction and characterization of *Streptomyces coelicolor* A3(2) mutants that are multiply deficient in the non-essential *hrd*-encoded RNA polymerase sigma factors. Mol. Microbiol. 3: 1653-1659, 1989.
 - 23) Brown, K.L.; S. Wood & M.J. Buttner: Isolation and characterization of the major vegetative RNA polymerase of *Streptomyces coelicolor* A3(2); renaturation of a sigma subunit using GroEL. Mol. Microbiol. 6: 1133-1139, 1992.
 - 24) Lange, R. & R. Hengge-Aronis: Identification of a central regulator of stationary phase gene expression in *Escherichia coli*. Mol. Microbiol. 5: 49-59, 1991.
 - 25) Chater, K. F.; C. J. Bruton, K. A. Plaskitt, M. J. Buttner, C. Mendez & J. D. Helmann: The developmental fate of *S. coelicolor* hyphae depends upon a gene product homologous with motility factor of *Bacillus subtilis*. Cell. 59: 133-143, 1989.
 - 26) Potůčková, L.; G. H. Kelemen, K. C. Findlay, M. A. Lonetto, M. J. Buttner & J. Kormanec: A new RNA polymerase sigma factor, σ^F , is required for the late stages of morphological differentiation in *Streptomyces* spp. Mol. Microbiol. 17: 37-48, 1995.
 - 27) Davis, N. K. & K. F. Chater: The *Streptomyces coelicolor* *whiB* gene encodes a small transcription factor-like protein dispensable for growth but essential for sporulation. Mol. Gen. Genet. 232: 351-358, 1992.
 - 28) Lonetto, M. A.; K. L. Brown, K. E. Rudd & M.J. Buttner: Analysis of the *Streptomyces coelicolor* *sigE* gene reveals the existence of a subfamily of eubacterial σ factors involved in the regulation of extracytoplasmic function. Proc. Natl. Acad. Sci. USA 174: 3843-3849, 1992.
 - 29) Fujii, T.; H.C. Gramajo, E. Takano & M.J. Bibb: *redD* and *actII-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} . J. Bacteriol. 178: 3402-3405, 1996.
 - 30) Wright, F. & M. J. Bibb: Codon usage in the G+C-rich *Streptomyces* genome. Gene 113: 55-65, 1992.

- 31) Lezhava, A.; T. Mizukami, T. Kajitani, D. Kameoka, M. Redenbach, H. Shinkawa, O. Nimi & H. Kinashi: Physical map of the linear chromosome of *Streptomyces griseus*. J. Bacteriol. 177: 6492-6498, 1995.
- 32) Lonetto, M.; M. Gribskov & C. A. Gross: The σ^{70} family: Sequence conservation and evolutionary relationships. J. Bacteriol. 174: 3843-3849, 1992.