Three Chymotrypsin Genes Are Members of the AdpA Regulon in the A-Factor Regulatory Cascade in *Streptomyces griseus*

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AdpA is a key transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*, activating a number of genes required for secondary metabolism and morphological differentiation. Of the five chymotrypsin-type serine protease genes, *sprA*, *sprB*, and *sprD* were transcribed in response to AdpA, showing that these protease genes are members of the AdpA regulon. These proteases were predicted to play the same physiological role, since these protease genes were transcribed in a similar time course during growth and the matured enzymes showed high end-to-end similarity to one another. AdpA bound two sites upstream of the *sprA* promoter approximately at positions -375 and -50 with respect to the transcriptional start point of *sprA*. Mutational analysis of the AdpA-binding sites showed that both AdpA-binding sites were essential for transcriptional activation. AdpA bound a single site at position -50 in front of the *sprB* promoter and greatly enhanced the transcription of *sprB*. The AdpA-binding site at position -40 was essential for transcription of *sprD*, although there was an additional AdpA-binding site at position -180 . Most chymotrypsin activity excreted by *S. griseus* was attributed to SprA and SprB, because mutant $\Delta sprAB$, having a deletion in both *sprA* and *sprB*, lost almost all chymotrypsin activity, as did mutant $\Delta adpA$. Even the double mutant $\Delta sprAB$ and triple mutant $\Delta sprABD$ grew normally and developed aerial hyphae and spores over the same time course as the wild-type strain.

A-factor (2-isocaprolyl-3R-hydroxymethyl- γ -butyrolactone) is a chemical signaling molecule, or a microbial hormone, that triggers secondary metabolism and cell differentiation in *Streptomyces griseus* (7, 8). We have revealed the A-factor regulatory cascade, through which production of almost all the secondary metabolites produced by *S. griseus* and formation of aerial mycelium and spores are triggered. A-factor is gradually accumulated in a growth-dependent manner, with its maximum quantity, about 25 ng/ml, at or near the decision point (1). The decision point is at the middle of the exponential growth phase (20). When the concentration of A-factor reaches a critical level, it binds ArpA, which has bound the promoter of *adpA*, and dissociates the ArpA from the DNA, thus inducing the transcription of *adpA* (7, 22). The transcriptional factor AdpA activates a number of genes required for secondary metabolism and morphological differentiation. Members of the AdpA regulon include *strR*, the pathway-specific transcriptional activator for streptomycin biosynthesis (30); an open reading frame encoding a probable pathway-specific regulator for a polyketide compound (35); *adsA* encoding an extracytoplasmic function sigma factor of RNA polymerase essential for aerial mycelium formation (32); *ssgA* encoding a small acidic protein essential for spore septum formation (33); and *amfR* encoding a regulatory protein essential for aerial mycelium formation (34). In addition to these genes, *sgmA* encoding a metalloendopeptidase (12) and *sprT* and *sprU*, both encoding a trypsin-type protease (9), are also

members of the AdpA regulon. Disruption of *sgmA* results in a delay of aerial mycelium formation, suggesting that it is involved in the lysis of substrate mycelium during aerial mycelium development. Disruption of *sprT* or *sprU* exerted no detectable phenotypic changes on the host.

S. griseus is known to produce many extracellular proteins, including SprT and SprU. Because *sprT*, *sprU* and *sgmA* are under the control of A-factor, we expected that other extracellular protease genes would be also controlled by A-factor. In addition to the three proteases, *S. griseus* produces five serine proteases, SGPA (*S. griseus* protease A) to SGPE. All the five genes, *sprA* encoding SGPA (6), *sprB* (6), *sprC* (26), *sprD* (27), and *sprE* (25, 36), have already been cloned and sequenced. SGPA and SGPB were purified from the commercially available protease mixture (Pronase), and subsequently their genes were cloned. The SGPC and SGPD genes were cloned on the basis of their sequence similarity to *sprB*. The SPGE gene was cloned and characterized because of the unique property of SPGE, which cleaves peptide bonds at the C side of glutamic acid. These proteases are all similar to mammalian chymotrypsin in their tertiary structure.

In the present study, we determined the dependence of the five chymotrypsins on AdpA. Of the five genes, *sprA*, *sprB*, and *sprD* were dependent on AdpA; AdpA bound their upstream activation sequences (UASs) and switched on their transcription. Contrary to our expectation, however, disruption of these genes gave no detectable phenotypic changes in morphological differentiation. During the study, we also found that a major extracellular protease(s) hydrolyzing skim milk supplemented to agar medium was under the control of AdpA. We will discuss a possible role of the proteases, whose production is switched on by AdpA, in morphological differentiation.

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TABLE 1. Primers used in this study

Gene	Primer	Positions ^a	Sequence (5' to 3')
<i>sprA</i>	A1-F	-77 to -55	GTCGACCCCCATCTCATTCCGGG
	A1-R	+243 to +223	CCTTGGACTCCGCTCGGGAG
	A2-F	-340 to -321	GCCGGGAAGCCGGCGATCAA
	A2-R	+127 to +105	GAGAAGCGCTTGAAGGTCACGAG
	A3-F	-496 to -476	GTGGACCGGCTACACACGCCG
	A3-R	-76 to -97	ACGAGTGGGGGTAGACCTGATC
	A4-F	+7 to +28	AACTTGGCAGGTTACGCCCCAC
	A5-R	-266 to -286	TTCCATCCCTCGCGCGGGGTC
	A6-F	-425 to -404	CTTCTTCCCAGGCGGAAGCACG
	A6-R	-163 to -184	TCGTGCTCAGGTGTGGCACCTG
	A8-F	-116 to -93	GGGTCAATTGATCGAGATGGATCA
	A8-R	+130 to +110	GGCGAGAAGCGCTTGAAGGTC
	AHs1-F	-135 to -112	CGCACCAAATTTTCTTTGGGGTC
	AHs1-R	+68 to +48	AGGTGGGGCCGTTGGTGCGCG
	AfAa-F	-182 to -161	GGTGCCACACCTGAGCACGACG
	AfAa-R	+27 to +5	TGGGCGTGAACCTGCCAAGTTGC
	AfAs-F	-111 to -86	AATTGATCGAGATCAGGTCTAC
	AfBs-F	-466 to -445	ACAAGAGAGCGTCTCCGGACGC
<i>sprB</i>	B1-F	-214 to -191	TTCGCCGATCACTTCATCTGCCCG
	B1-R	+266 to +246	TCGCCGCGGTCACTGGTTGG
	B2-F	-44 to -23	GGCATTTCTTGCCTCCCCCGTCC
	B3-R	-35 to -57	CAAGAATGCCTTTCGGCCACGCG
	B4-R	+107 to +86	CGGGTTCTCCGAGGGGAATCG
	B5-F	-134 to -115	GATCGACGCCTGACCCGCGC
	B6-R	-125 to -145	GGCGTCGATCCGGTTCCGCTC
	B7-F	-323 to -301	GACCAGCGGAAACAGCAGCTACC
	Bs1-F	-144 to -125	AGCGGAACCGGATCGACGCC
	Bs1-R	+323 to +302	ACTGCGGGTCGATGTTCCAGGC
	Bfa-R	+13 to -9	CCCTGACAATCGCTGACCGGAG
	Bfs-R	+78 to +59	TCCTGTGGGGTGGCGGGCCC
<i>sprC</i>	Cgs-F	-380 to -360	CCCGGTAACGGCGAAGGACCC
	Cgs-R	+20 to -1	CGGAGCGTGGTTCTCTCCACG
<i>sprD</i>	D1-F	-192 to -172	GATCCGCGCCGTCGAATACCG
	D1-R	+220 to +201	CAGCTTTCCGGCCGCGTCCG
	D2-F	-143 to -122	TCAACGGTCGCACCGAGTGTC
	D3-F	-94 to -75	GGGGAGGTGAACGCCTCGCG
	D4-F	-43 to -21	AAGTCGACCTTGTGTGCGTTTCG
	D5-F	-390 to -370	ACCCGATTGCGCACCCGTTCC
	D5-R	-22 to -44	GAAACGCACACAAGGTCGACTTC
	D6-R	-75 to -94	CGCGAGGCGTTACCTCCCC
	D7-R	-123 to -144	AACACTCGGTGCGACCGTTGAC
	D8-R	-172 to -192	CGGTATTGACGCGCGCGGATC
	DHs1-R	+95 to +74	TCGCGCGCTTCTGGATATGCG
	Dms1-R	+252 to +232	TCCGCGCCAGGTACACGATCG
	DfAa-R	+17 to -4	GTACACGGAGGATGGGGCGCC
	DfBa-F	-273 to -253	GGCGACAGGAAGTGTGCGGGG
	DfBs-F	-233 to -213	ACGATTCGCCGCGGCATCCCC
<i>sprE</i>	Egs-F	-355 to -334	ACGGTCCTTCGCTCGTCCATGC
	Egs-R	+50 to +29	GCGACGAGCAGCAGGGAAACAC

^a The nucleotide positions for *sprA*, *sprB*, and *sprD* are given, taking their transcriptional start points as +1. Those for *sprC* and *sprE* are given, taking the first nucleotides of their start codons as +1.

MATERIALS AND METHODS

General recombinant DNA studies. All the bacterial strains, plasmids, and media were previously described (9, 30). The strategies of gene disruption, alteration of the AdpA-binding sites by PCR, gel mobility shift assaying, DNase I footprinting, and S1 nuclease mapping were also previously described (30, 32).

Cloning of the DNA fragments containing the protease genes. We cloned four protease genes from *S. griseus* IFO13350, on the basis of the reported nucleotide sequences of *sprA* (DNA database accession number A24972), *sprB* (A24973), *sprD* (L29019) (27), and *sprE* (L28762). The strategy was first to amplify a short region by PCR with appropriate primers and then to clone a long DNA fragment containing the protease gene by colony hybridization with the amplified region as

a digoxigenin-labeled probe. By this strategy, *sprA* on an 11-kb PvuII-BamHI fragment, *sprB* on an 8.6-kb BamHI fragment, *sprD* on a 6.7-kb BglII-SphI fragment, and *sprE* on a 4.3-kb SphI fragment were cloned. Concerning *sprC* (AF515832), the nucleotide sequence in the database was sufficient for our gel mobility shift assay with AdpA, and we amplified *sprC* sequences by PCR by using the chromosomal DNA of *S. griseus* IFO13350, without cloning *sprC*.

Gel mobility shift assay. The DNA fragments used for ³²P-labeled probes were amplified by PCR and ³²P labeled with T4 polynucleotide kinase. Various regions upstream of the coding sequences of *sprA* to *sprE* were used as ³²P-labeled probes. Table 1 lists the primer sequences for preparing these probes. Primers and seven probes, A1 to A7, were prepared for *sprA*: primers A1-F and A1-R for

FIG. 1. Gel mobility shift assays to determine AdpA binding to regions upstream of *sprD*. Binding of AdpA-H to the regions of *sprD* was determined by gel mobility shift assays with eight probes, D1 to D8. The probes yielding a positive signal(s) are shown by gray bars, and the probes yielding no signal are shown by open bars. Predicted AdpA-binding sites are shown by solid bars. The amounts of AdpA-H used were 0 μ g (lane 1), 0.1 μ g (lane 2), 0.2 μ g (lane 3), and 0.4 μ g (lanes 4 to 6). Lanes 5 and 6 show competition of binding between the probe and AdpA-H by an excess of nonlabeled probe (lane 5, $\times 50$; lane 6, $\times 100$). Two shifted signals, indicated by arrowheads, are seen in lane 4.

probe A1; A2-F and A2-R for probe A2; A3-F and A3-R for probe A3; A4-F and A1-R for probe A4; A3-F and A5-R for probe A5; A6-F and A6-R for probe A6; and A2-F and A3-R for probe A7. Primers and eight probes were prepared for *sprB*: primers B1-F and B1-R for probe B1; B2-F and B1-R for probe B2; B1-F and B3-R for probe B3; B1-F and B4-R for probe B4; B5-F and B4-R for probe B5; B1-F and B6-R for probe B6; B7-F and B6-R for probe B7; and B7-F and B3-R for probe B8. The following primers and probes were prepared for *sprD* (Fig. 1, left panel): primers D1-F and D1-R for probe D1; D2-F and D1-R for probe D2; D3-F and D1-R for probe D3; D4-F and D1-R for probe D4; D5-F and D5-R for probe D5; D5-F and D6-R for probe D6; D5-F and D7-R for probe D7; and D5-F and D8-R for probe D8. For *sprC*, a 0.4-kb fragment (nucleotide positions -380 to $+20$, taking the first letter of the translational start codon of *sprC* as $+1$) was amplified by PCR with primers Cgs-F and Cgs-R and used as the probe. For *sprE*, a 0.4-kb fragment (positions -355 to $+50$, taking the first letter of the translational start codon of *sprE* as $+1$) was amplified by PCR with primers Egs-F and Egs-R and used for the probe.

S1 nuclease mapping. Total RNA was isolated with ISOGEN (Nippon Gene) from cells grown on cellophane on the surface of YMPD agar medium. Hybridization probes were prepared by PCR with a pair of 32 P-labeled and nonlabeled primers. The PCR primers used for low-resolution S1 mapping were A1-F and A1-R* for *sprA*, B51-F and B51-R* for *sprB*, and D1-F and D1-R* for *sprD*. Primers indicated with an asterisk were labeled at their 5' ends with [γ - 32 P]ATP by using T4 polynucleotide kinase before PCR. For the *adpA* probe, 5'-AGCC CCCGATCCCTCCGCGGCGA-3' (positions -223 to -200 with respect to the transcriptional start point of *adpA*) and 5'-ACTCGCGAAGCGCACAGG GAAGTG-3' ($+54$ to $+31$) were used. Primers used for high-resolution S1 mapping were AHs1-F and AHs1-R* for *sprA*, B5-F and B4-R* for *sprB*, and D3-F and DHs1-R* for *sprD*.

For detection of the transcription of *sprA* on plasmids pA-W, pA-mA, pA-mB, and pA-mAB, the probe with 32 P at the 5' end, which was deleted from the chromosome of mutant Δ *sprA*, was prepared with primers A1-F and A1-R*. For detection of the *sprB* transcription on plasmids pB-W and pB-M, the probe was prepared with B51-F and B51-R*. For detection of the *sprD* transcription on plasmids pD-W, pD-mA, pD-mB, and pD-mAB, the probe was prepared with primers D1-F and Dms1-R*. *hrdB* encoding a principal sigma factor of RNA polymerase was used to check the purity and amount of RNA used, as described previously (23). Protected fragments were analyzed on 6% polyacrylamide DNA sequencing gels by the method of Maxam and Gilbert (18).

DNase I footprinting. For determination of the AdpA-binding sites, a 32 P-labeled fragment was prepared by PCR with a pair of 32 P-labeled (indicated with an asterisk) and unlabeled primers listed in Table 1: AfAa-F and AfAa-R* for the antisense strand and AfAs-F* and A2-R for the sense strand of site A in front of *sprA*; A3-F and A5-R* for the antisense strand and AfBs-F* and A6-R for the sense strand of site B in front of *sprA*; B1-F and Bfa-R* for the antisense strand and B51-F* and Bfs-R for the sense strand of AdpA-binding site in front of *sprB*; D1-F and DfAa-R* for the antisense strand and D3-F* and DHs1-R for the sense strand of site A in front of *sprD*; and DfBa-F and D6-R* for the antisense strand and DfBs-F* and D5-R for the sense strand of site B in front of *sprD*.

Alterations of the AdpA-binding sequences by PCR. Mutations were introduced in the AdpA-binding sites of *sprA*, *sprB*, and *sprD* by PCR using the

QuikChange multisite-directed mutagenesis kit (Stratagene). For introduction of a mutation into the two AdpA-binding sites in front of *sprA* (see Fig. 6A), a 0.7-kb fragment (positions -610 to $+130$) was amplified by PCR with primers 5'-GGAATTCCGAGGCTGCGGACGGGTC-3' (positions -610 to -591 , with respect to the transcriptional start point of *sprA*, which was later determined; the underline indicates an EcoRI site) and 5'-CGGGATCCGGCGAG AAGCGCTTGAAGGTC-3' (positions $+130$ to $+110$; the underline indicates a BamHI site). After the absence of PCR errors had been checked by nucleotide sequencing, the fragment was cloned into pKF18k, resulting in pKFA. A mutation was introduced in the AdpA-binding sequence in site A on pKFA by PCR with primer 5'-CGGGCTCGCGGGCagcgtCCGGCCTTGCCTC-3' (the lowercase letters indicate an Aor51HI site). A mutation was introduced in each of the four AdpA-binding sequences in site B, in the order of the XhoI, SacI, PstI, and PmaCI mutations (see Fig. 6A), on pKFA by PCR with primers 5'-TTCA GCCAGCCctcgagGTAGCAAGTACGC-3' (positions -401 to -371 ; the lowercase letters indicate an XhoI site), 5'-AGCAAGTACGCCagctcCACCATG GCTC-3' (positions -381 to -353 ; the lowercase letters indicate a SacI site), 5'-CCTCGAGGTAGCctcgagGCCGAGCTCCAC-3' (positions -390 to -361 ; the lowercase letters indicate a PstI site and the underline indicates the nucleotides that have already been changed), and 5'-CGGAAGCACGTAcagtgCA GCCCCTCGAG-3' (positions -413 to -384 ; the lowercase letters indicate a PmaCI site and the underline indicates the nucleotides that have already been changed). Thus, the mutation in site B contained the four newly introduced restriction sites. The 32 P-labeled probes used for gel mobility shift assays to determine the affinity of the mutated sites were prepared by PCR with A8-F and A8-R for site A and with A3-F and A5-R for site B. The mutations in sites A and B, together with the whole *sprA*-coding sequence, were used for reconstruction of the 1.9-kb EcoRI-BamHI fragment (nucleotide positions -610 to $+1319$) in pUC19, and the fragment was placed between the BamHI and EcoRI sites of pKUM20. The plasmids constructed in this way were pA-W containing the intact AdpA-binding sites, as a control; pA-mA containing the mutated site A and intact site B; pA-mB containing the intact site A and mutated site B; and pA-mAB containing the mutated sites A and B.

For generation of an XhoI site in the AdpA-binding site in *sprB*, a region (positions -214 to $+77$, with respect to the transcriptional start point of *sprB*) was amplified by PCR with primers 5'-CGGGATCCTTCGCCGATCACTTCA TCTGCC-3' (positions -214 to -193 ; the underline indicates a BamHI site) and 5'-CCCAAGCTTCTGTGGGGTGGCGGGCC-3' (positions $+77$ to $+59$; the underline indicates a HindIII site). After confirmation that no errors had occurred during PCR, the BamHI-HindIII fragment was cloned in pKF18k. The XhoI mutation was introduced with a primer, 5'-GACCCGCTGGCctcgagGC ATTCTTGCCTCC-3' (positions -61 to -30 ; the lowercase letters an XhoI site), by the same method as that used for introduction of the mutations in the sites A and B of *sprA*. The 1.3-kb BamHI-EcoRI fragment, corresponding to the DNA region from -214 to $+1069$, was reconstructed and placed between the BamHI and EcoRI sites of pKUM20, resulting in pB-W containing the intact AdpA-binding site, as a control, and pB-M containing the mutated AdpA-binding site. The 32 P-labeled probe to check the affinity of the mutated site by gel mobility shift assay was prepared by PCR with primers B51-F and Bfs-R.

For generation of mutations in the two AdpA-binding sequences for *sprD* (see

Fig. 6A), the 0.9-kb BamHI-PvuII fragment containing the *sprD* promoter (nucleotide positions -193 to +702, with respect to the transcriptional start point of *sprD*) was cloned between the BamHI and PvuII sites of pKF18k. For generation of a PmaCI site in site A, 5'-TGCACGTCTGGAcacgtgCCTGTGTGCG-3' (positions -54 to -26, with respect to the transcriptional start point of *sprD*, which was later determined; the lowercase letters indicate a PmaCI site) was used. For generation of an Aor51HI site in site B, 5'-GATCCGCGCCGTagcgctCCGGACAGGACTAG-3' (positions -192 to -161; the lowercase letters indicate an Aor51HI site) was used. The ³²P-labeled probes for determination of the affinity of mutated sites for AdpA were prepared by PCR with primers D2-F and DHs1-R for site A and primers DfBa-F and D6-R for site B. By combination of the generated mutations by standard DNA manipulation, four plasmids containing the DNA region from -472 to +1482 between the EcoRI and XhoI sites of pKU209 were constructed: these were pD-W containing the intact sites A and B, as a control; pD-mA containing the mutated site A and the intact site B; pD-mB containing the intact site A and the mutated site B; and pD-mAB containing the mutated sites A and B.

Gene disruption. For disruption of *sprA*, an EcoRI-PvuII fragment, from which a region corresponding to Met-1 to Ala-294 was deleted, was cloned on pUC19 by DNA manipulation (see Fig. 7A). The neomycin resistance gene (*aphII*) was placed in the multicloning site of the pUC19 plasmid. This mutagenic plasmid was introduced by transformation in *S. griseus* IFO13350, and neomycin (5 µg/ml)-resistant colonies were isolated. After one of the neomycin-resistant transformants had been cultured in the absence of neomycin, neomycin-sensitive colonies were isolated as candidates for true *sprA* disruptants (mutant Δ *sprA*). Correct disruption was checked by Southern hybridization with appropriate regions as ³²P-labeled probes.

For disruption of *sprB*, the Sall-BamHI fragment, from which the PvuII-SmaI region was deleted, was placed in pUC19 and *aphII* was also placed in the HindIII site at the multilinker site. This plasmid was used as a mutagenic plasmid, and correct *sprB* disruptants (mutant Δ *sprB*) were similarly isolated after Southern hybridization to check the correct deletion in the *sprB*-coding sequence.

For disruption of *sprD*, the Ball-PvuII fragment, from which the Ball-PvuII region in the *sprD*-coding sequence was deleted, was placed in pUC19, together with *aphII* in the multilinker site. This plasmid was used as a mutagenic plasmid, and correct mutant Δ *sprD* was isolated after Southern hybridization.

For construction of double mutant Δ *sprAB*, *sprA* in mutant Δ *sprB* was disrupted. For construction of triple mutant Δ *sprABD*, *sprD* in mutant Δ *sprAB* was disrupted.

Serine protease assay. *S. griseus* strains were grown at 28°C for various periods on YMPD agar in a petri dish. Proteases secreted in the agar were extracted by a passage through the nozzle of a 20-ml plastic syringe without needle. The resulting suspension was centrifuged at 10,000 × g at 4°C for 30 min for removal of agar debris. The supernatant was filtered through a Millex-GV filter (Millipore) (0.2-µm diameter) to eliminate the remaining agar. The chymotrypsin activity was measured spectrophotometrically by the release of *p*-nitroaniline by use of *N*-succinyl-L-alanyl-L-proline-*p*-nitroanilide (AAPF; Sigma) as an artificial chromogenic substrate (17). The reaction mixture containing 890 µl of a reaction buffer (100 mM Tris-HCl [pH 8.0], 10 mM CaCl₂) and 10 µl of 30 mM AAPF in dimethyl sulfoxide was warmed at 37°C for 5 min, rapidly mixed with 100 µl of the enzyme solution, and incubated for 15 min. The reaction was stopped by adding 400 µl of 30% acetic acid in dioxane, and the absorbance at 405 nm was recorded. One unit of hydrolytic activity was defined as the amount of enzyme corresponding to a 0.1 increase in absorbance per min under the above-described conditions.

RESULTS

Cloning of protease genes. The five proteases, SprA to SprE, are all chymotrypsin-type serine proteases (27). All consist of the pre-, pro-, and mature regions. Although the mature parts of these proteases show similarity in amino acid sequence to one another, SprA, SprB, and SprD show particularly high sequence similarity to one another. Therefore, these proteases might play the same physiological role, and each might complement the activity of the others. SprE is a glutamic acid-specific protease (25). On the basis of the nucleotide sequences registered in the NCBI sequence data bank, we cloned *sprA*, *sprB*, *sprD*, and *sprE*, the four serine protease genes, from *S.*

griseus IFO13350. We used the *sprC* sequence amplified by PCR with the chromosomal DNA as a template without cloning *sprC*. Because the cloned DNA fragments were later used for disruption of the chromosomal genes by replacement as a result of homologous recombination, long regions upstream and downstream of *sprA*, *sprB*, and *sprD* were cloned.

Detection of AdpA-binding sites upstream of *sprA*, *sprB*, and *sprD*. We determined whether AdpA binds regions upstream of the five genes by a gel mobility shift assay. AdpA activates target genes by binding various positions with respect to the transcriptional start point, for example, more than 200 bp upstream and 25 bp downstream from the transcriptional start point (35). In addition, some target genes contain multiple AdpA-binding sites; for example, *ssgA* contains three sites, from -255 to -216, from -127 to -95, and from +35 to +87 (35). We therefore designed ³²P-labeled probes covering various regions and used them for gel mobility shift assays. The AdpA protein used was purified from *E. coli* harboring pET-*adpA* and had a histidine tag at its C-terminal end with the structure AdpA-Leu-Glu-His₆ (32).

Preliminary experiments showed the binding of AdpA to the upstream regions of *sprA*, *sprB*, and *sprD*. We therefore designed seven probes for *sprA*, eight probes for *sprB*, and eight probes for *sprD* and performed gel mobility shift assays. Figure 1 shows the results of the gel mobility shift assays to determine the AdpA-binding sites in front of the *sprD* promoter. Probe D1 gave two shifted signals, indicating that AdpA bound two different sites on this probe. This analysis predicted that AdpA bound two sites, nucleotide positions -171 to -123 and positions -94 to -44, with respect to the transcriptional start point which was later determined by S1 mapping. Similar analysis of *sprA* and *sprB* predicted that AdpA bound two sites, from -496 to -341 and from -75 to +6, in front of *sprA* and one site, from -124 to -45, in front of *sprB*.

When the region from -380 to +20, taking the first letter of the translational start codon of *sprC* as +1, was used as a ³²P-labeled probe, no shifted signal was observed even with a large amount of AdpA (data not shown). Neither was any shifted signal observed for the *sprE* region from -355 to +50, taking the first letter of the translational start codon of *sprE* as +1 (data not shown).

Dependence of transcription of *sprA*, *sprB*, and *sprD* on AdpA. Because AdpA bound the region upstream of *sprA*, *sprB*, and *sprD*, we examined the transcription of these genes in the presence and absence of *adpA* (Fig. 2A). RNA was prepared from the wild-type strain IFO13350 and an *adpA* disruptant (Δ *adpA*) that were grown on cellophane on the surface of YMPD agar medium. *hrdB*, which is transcribed throughout growth, was used as an internal control to check the purity and amount of mRNA used. In the wild-type strain, the transcription of *sprA* and *sprB* was detected at 24 h, when cells vegetatively grew, and at 72 h, when sporulation began, as determined by low-resolution S1 mapping, whereas no transcription of *sprA* or *sprB* occurred in mutant Δ *adpA*. The transcription of *sprD* was observed at 24 h in the wild-type strain, whereas no transcription of *sprD* occurred in mutant Δ *adpA*. When *adpA* on a low-copy-number plasmid pKU209 (plasmid pADP10L) was introduced in mutant Δ *adpA*, the transcription of all the three genes at 24 h was recovered and enhanced. This enhancement is probably due to the gene dosage effect of *adpA*;

FIG. 2. Transcriptional analysis of *sprA*, *sprB*, and *sprD*. (A) The time courses of *sprA*, *sprB*, and *sprD* transcription were followed by low-resolution S1 mapping with RNA prepared from cells grown at 28°C for the indicated hours on cellophane on the surface of YMPD medium. As a control, transcription of *adpA* and *hrdB* was also determined. (B) The time course of *sprB* transcription was determined with RNA prepared at more points. (C) The transcriptional start points of *sprA*, *sprB*, and *sprD* were determined by high-resolution S1 mapping. RNA (40 µg each) prepared from the wild-type (wt) strain grown for 24 h on YMPD medium was used. The arrowheads indicate the positions of the S1-protected fragments. The 5' termini of the mRNA were assigned to the positions indicated by arrows, because the fragments generated by the chemical sequencing reactions migrate 1.5 nucleotides further than the corresponding fragments generated by S1 digestion of the DNA-RNA hybrids (half a residue from the presence of the 3'-terminal phosphate group and one residue from the elimination of the 3'-terminal nucleotide).

pKU209 appeared to have a copy number of more than 2, because introduction of pADP10L into mutant $\Delta adpA$ results in overproduction of streptomycin (23). These results showed that the transcription of *sprA*, *sprB*, and *sprD* at 24 h was dependent on *adpA*.

Introduction of *adpA* into mutant $\Delta adpA$ did not restore the transcription of *sprA* or *sprB* at 72 h, the reason for which is unclear. One speculation is that enhanced expression of *adpA* at 24 h in mutant $\Delta adpA$ harboring pADP10L somehow extends the period of transcriptional repression observed at 48 h in the wild-type strain until 72 h. In the wild-type strain, the cease of the transcription of *sprA* and *sprB* continued for a long period, more than 24 h, when examined with RNA prepared at more points (Fig. 2B). The mechanism of the repression of *sprA* and *sprB* in the wild-type strain is also unclear.

The transcriptional start points of *sprA*, *sprB*, and *sprD* were determined to be 107, 109, and 61 nucleotides, respectively, upstream of the translational start codon, as determined by high-resolution S1 mapping (Fig. 2C). The consensus -35 and -10 sequences are TTGACR and TAGRRT (R; A or G), respectively, for housekeeping genes in *Streptomyces* species (28). Probable -35 and -10 sequences at appropriate positions for these genes are shown in Fig. 3. Both elements of these genes are similar to one another and show 50% identity to those of the housekeeping genes. Two additional trypsin-

type protease genes, *sprT* and *sprU*, both of which are activated by AdpA (9), also contain -35 and -10 sequences similar to those of the chymotrypsin-type protease genes (Fig. 3). We can deduce consensus sequences, ccTTG(C/T)G(T/G)c (the six nucleotides usually used as a -35 element are in uppercase letters) for -35 and c(G/A)A(T/C)AAT for -10. It is likely that the major sigma factor HrdB (2) is involved in the transcription of these promoters.

DNase I footprinting analysis of the AdpA-binding sites upstream of *sprA*, *sprB*, and *sprD*. We determined the exact location of the AdpA-binding sites by DNase I footprinting (Fig. 4). For the antisense strand in site A of *sprA*, a 32 P-labeled probe covering the region from -182 to +27 was used. AdpA protected a sequence from positions -32 to -58 with respect to the transcriptional start point. The sense strand from -64 to -29 was protected from DNase I digestion when 32 P-labeled probe from -111 to +127 was used. For the antisense strand in site B of *sprA*, a 32 P-labeled probe covering the region from -496 to -266 was used. A sequence from -368 to -404 was protected. The sequence from -349 to -367 was also weakly protected. When a 32 P-labeled probe from -466 to -163 was used, the sense strand from -401 to -379 was protected. The AdpA-binding sites, with respect to the transcriptional start point, are shown in Fig. 5.

The single AdpA-binding site in front of *sprB* was analyzed

FIG. 5. The promoters and AdpA-binding sites upstream of *sprA*, *sprB*, and *sprD*. The transcriptional start point (indicated by an arrow with +1) and AdpA-binding site(s) of *sprA* (A), *sprB* (B), and *sprD* (C) are shown. AdpA-binding sequences are shown by boldface letters in the regions protected from DNase I digestion (see Fig. 4). The AdpA-binding sites, relative to the transcriptional start points, together with the presumptive manner of AdpA binding, are shown (D).

The consensus AdpA-binding sequence in the AdpA-binding sites. The AdpA-binding sites in front of *sprA*, *sprB*, and *sprD* are shown in Fig. 5. The consensus AdpA-binding sequence is 5'-TGGCSNGWWY-3' (S, G or C; W, A or T; Y, T or C; N,

any nucleotide) (35). Site A of *sprA* contains a single AdpA-binding consensus sequence, 5'-GGGCGCGAAT-3'. The AdpA-binding site for *sprB* and the two AdpA-binding sites A and B for *sprD* also contain a single AdpA-binding sequence.

A

B

C

AdpA binds the sites of this type (type II) by anchoring the DNA via the two DNA-binding motifs in one subunit of an AdpA dimer (24, 35).

Site B of *sprA* contains two consensus AdpA-binding sequences, 5'-TGGCTGAATA-3' and 5'-TAGCAAGTAC-3', as a divergent repeat with a space of 11 bp. This pair seems to serve as the major AdpA-binding site, although this site contains an additional pair of the AdpA-binding sequences, as described below. This is a typical type I AdpA-binding site, to which AdpA binds by anchoring one AdpA-binding sequence with the DNA-binding domain of one subunit and the other AdpA-binding sequence with the other DNA-binding domain of the other subunit (35). The AdpA-binding sites for *sprA*, *sprB*, and *sprD*, relative to their transcriptional start points, are illustrated in Fig. 5D. Most target genes activated by AdpA contain one AdpA-binding site at position -40 to -50 with the same orientation of the recognition sequence, 5' to 3', as that of transcription. The AdpA dimer bound to this site is predicted to recruit RNA polymerase to initiate transcription. In fact, AdpA facilitates RNA polymerase to form an open complex competent for transcriptional initiation at the promoters of *strR* (30), *ssgA* (35), and *adsA* (35).

Alterations of the AdpA-binding sequences. We introduced a mutation in the AdpA-binding sequences of *sprA*, *sprB*, and *sprD* by site-directed mutagenesis to determine the importance of the AdpA-binding sequences and measured the effects of the mutation on the promoter activity to later determine whether AdpA directly controls the transcription of these genes. As shown in Fig. 6A, the six nucleotides in the consensus AdpA-binding sequence in site A for *sprA* were changed to an Aor51HI recognition sequence of six nucleotides. The ³²P-labeled probe (positions -116 to +130) containing the Aor51HI mutation and the promoter of *sprA* gave almost no retarded signal, whereas a similar probe containing the intact AdpA-binding sequence gave a distinct signal. Similarly, the single AdpA-binding sequence for *sprB* was changed to produce an XhoI recognition sequence, site A for *sprD* was changed to produce a PmaCI recognition sequence, and site B for *sprD* was changed to produce an Aor51HI recognition sequence. Gel mobility shift assays with a ³²P-labeled probe (positions -144 to +78) containing the XhoI mutation and the *sprB* promoter gave no retarded signal, whereas a similar probe containing the intact AdpA-binding sequence gave a distinct signal. Gel mobility shift assays with ³²P-labeled probes (positions -143 to +95 for site A of *sprD* and from -273 to -75 for site B of *sprD*) containing the PmaCI and Aor51HI mutations,

respectively, did not give retarded signals, whereas similar probes containing the intact AdpA-binding sequences gave distinct retarded signal.

Site B for *sprA* contained two pairs of the type II AdpA-binding site. In addition to the pair described above, 5'-CTA CCTTTC-3' and 5'-GTACGCCATT-3' formed a divergent repeat with a space of 3 bp (Fig. 6A). We first generated the XhoI mutation and examined the affinity for AdpA by using a ³²P-labeled probe (positions -496 to -266) containing the XhoI mutation by a gel mobility shift assay. However, AdpA gave almost the same signal as for a similar probe containing no mutation (data not shown). We further added the SacI mutation to the XhoI mutation and examined the affinity of the probe containing the XhoI and SacI mutations. AdpA still gave a significant retarded signal. We next added the PstI mutation and examined the affinity containing the triple mutations. The affinity of this fragment for AdpA was greatly reduced. Finally, we added the PmaCI mutation. The ³²P-labeled probe containing the XhoI, SacI, PstI, and PmaCI mutations gave a negligible signal (Fig. 6B). Because the XhoI and SacI mutations resulted in only slight decreases in affinity for AdpA and because the PstI and PmaCI mutations resulted in significant decreases in the affinity, we assume that the pair of the AdpA-binding sequences, positions -403 to -394 and positions -382 to -373, serves as the major AdpA-binding site.

Importance of the AdpA-binding sites for transcriptional activation of *sprA*, *sprB*, and *sprD*. We constructed pA-W, containing a 1.9-kb fragment in which the *sprA* promoter, together with its upstream region as far as position -610, and the complete *sprA*-coding sequence were contained, with the low-copy-number plasmid pKUM20 (Fig. 6C). We also constructed similar pA-series plasmids: these were pA-mA, in which the AdpA-binding site A was replaced by the Aor51HI mutation; pA-mB, in which site B was replaced by the XhoI/SacI/PstI/PmaCI mutation; and pA-mAB, in which both mutations at sites A and B were combined. These plasmids were introduced in mutant $\Delta sprA$, and the transcription from the *sprA* promoter on the plasmids was measured. Because the ³²P-labeled end of the probe used for S1 mapping was deleted from the chromosome, the transcript detected should originate from the *sprA* promoter on the plasmids. The *sprA* transcript in mutant $\Delta sprA$ harboring pA-W was detected at 36, 72, and 108 h, whereas no *sprA* transcripts were detected in mutant $\Delta sprA$ harboring the other pA-series plasmids. Therefore, AdpA directly controlled the *sprA* transcription and both sites A and B were essential for the transcriptional activation.

FIG. 6. Effects of mutations in the AdpA-binding sequences for *sprA*, *sprB*, and *sprD* on the promoter activities. (A) Mutations were introduced in the AdpA-binding sequences by PCR to produce recognition sequences of restriction enzymes. The consensus AdpA-binding sequences are indicated by lines with nucleotide numbers. The nucleotides changed are shown in boldface letters. (B) Gel mobility shift assays for determination of AdpA binding to the mutated sequences. For example, the ³²P-labeled probe containing the intact site A of *sprA* (shown as lanes A) yields a distinct shifted signal, whereas a similar probe containing the mutated site A of *sprA* (shown as lanes mA) did not. The ³²P-labeled probes used were positions -116 to +130 for site A of *sprA*, -496 to -266 for site B of *sprA*, -144 to +78 for the single site of *sprB*, -143 to +95 for site A of *sprD*, and -273 to -75 for site B of *sprD*. (C) Transcriptional analysis of the promoters containing the mutated AdpA-binding sequences. For example, pA-W, as a control, contained the *sprA*-coding region and the intact AdpA-binding sequences on pKUM20, and pA-mA contained the mutated AdpA-binding sequence. Low-resolution S1 mapping was performed with RNA prepared from cells of mutant $\Delta sprA$ harboring pA-series plasmids grown at 28°C for the indicated hours on YMPD agar medium. No transcription from the *sprA* promoter with the mutated site A (pA-mA) was observed, whereas distinct *sprA* transcripts from the *sprA* promoter with the intact AdpA-binding sequences (pA-W) were detected.

FIG. 7. Deletion of the chromosomal *spr* genes and chymotrypsin activities of the mutants. (A) Schematic representation of deletion of *sprA* on the chromosome of *S. griseus* IFO13350, as an example. For generation of mutant $\Delta sprA$, long DNA fragments upstream and downstream were combined so that most *sprA*-coding sequence was

Plasmids pB-W and pB-M were constructed to assess the importance of the AdpA-binding site upstream of *sprB* (Fig. 6C). The *sprB* transcript from the *sprB* promoter on pB-W was detected at 36 and 72 h, whereas that from the *sprB* promoter with the XhoI mutation at the AdpA-binding sequence was greatly reduced. We concluded that the control of the *sprB* promoter by AdpA was not as strict as for the *sprA* promoter but that AdpA was important for transcriptional enhancement of *sprB*.

pD-series plasmids were used for determination of the importance of sites A and B for transcriptional activation of *sprD* by AdpA. The amount of the *sprD* transcript from the *sprD* promoter with the Aor51HI mutation at site B was almost the same as that from the wild-type promoter. The *sprD* transcription from the *sprD* promoter with the PmaCI mutation at site A and with the mutation at both sites A and B was almost abolished. These results showed that only site A was responsible for the transcriptional activation of *sprD* by AdpA.

Disruption of the protease genes. We disrupted *sprA*, *sprB*, and *sprD* to determine their possible roles in morphological development. Figure 7A illustrates an example to show the strategy for disruption of *sprA*. Mutant $\Delta sprA$ contained an in-frame deletion of the region encoding from Met-1 to Ala-294, mutant $\Delta sprB$ contained an in-frame deletion of the region encoding from Leu-49 to Arg-264, and mutant $\Delta sprD$ contained an in-frame deletion of the region encoding from Ala-85 to Ser-244. Because the activity of each of these proteases appeared to compensate for their roles by that of the others, we also generated a double mutant, $\Delta sprAB$, and a triple mutant, $\Delta sprABD$.

The extracellular chymotrypsin activities of the mutants grown on YMPD agar medium were compared to that of the wild-type strain (Fig. 7B). The chymotrypsin activities of mutants $\Delta sprA$, $\Delta sprB$, and $\Delta sprD$ decreased throughout the growth. Because mutant $\Delta sprAB$, like mutant $\Delta adpA$, showed almost no chymotrypsin activity, almost all chymotrypsin activity in *S. griseus* was attributed to SprA and SprB. Introduction of pADP10L containing *adpA* on the low-copy-number plasmid pKU209 into mutant $\Delta adpA$ restored the chymotrypsin activity (Fig. 7C), indicating again that these protease genes are under the control of *adpA*. The chymotrypsin activities of the wild-type strain and mutant $\Delta adpA$, both harboring the

deleted and were placed next to the kanamycin resistance (*aphII*) gene in pUC19. The pUC19 plasmid was introduced in *S. griseus* to isolate kanamycin-resistant transformants, in which the whole plasmid was integrated by homologous recombination. After cultivation of one of the kanamycin-resistant transformants in the absence of kanamycin, candidates having the correct deletion were selected as kanamycin-sensitive colonies. Correct mutants ($\Delta sprA$) were selected by Southern hybridization with *aphII* and the DNA fragment, shown by a bar, as the 32 P-labeled probes. Mutants $\Delta sprB$ and $\Delta sprD$ were similarly constructed. Mutant $\Delta sprB$ was first constructed and then the *sprA* locus in this mutant was deleted, generating mutant $\Delta sprAB$. The *sprD* locus on the chromosome of mutant $\Delta sprAB$ was deleted, generating mutant $\Delta sprABD$. (B) Extracellular chymotrypsin activities of the mutants as a function of cultivation time. Strains were grown at 28°C on YMPD agar medium. Each point is a mean of the values obtained from three independent experiments. (C) Restoration of chymotrypsin activity in mutant $\Delta adpA$ by *adpA* on pADP10L. Chymotrypsin activities were measured as described for panel B. wt, wild type.

FIG. 8. Hydrolysis of skim milk in agar medium by *S. griseus* strains. *S. griseus* IFO13350 (wt) and three mutants were grown at 28°C for 4 days on Bennett-maltose agar containing 1% skim milk.

vector pKU209, were higher than those of the strains without the vector. This may be due to the thiostrepton supplemented to the medium for the vector-containing strains, which might change physiological conditions in some way.

Proteases have been suggested to be important for morphological differentiation, particularly in aerial mycelium formation (14–17). We expected that these proteases, which are all controlled by A-factor, might exert some effects on morphogenesis. All these mutants were grown on various media as a lawn by spreading a lump of mycelium and as a colony by inoculating a lump of mycelium or spores with a toothpick. The media we tested were YMPD, R2YE, Trypto-Soya, and minimal media. The carbon source (glucose) of these media was also changed to mannitol and glycerol. Contrary to our expectation, however, no apparent phenotypic changes were observed in the mutants; these mutants grew normally and formed aerial hyphae and spores with the same time course as the wild-type strain.

Dependence of extracellular protease activity on AdpA. We assayed extracellular protease activities by measuring the size of clear zones formed around the colonies of mutants $\Delta sprA$, $\Delta sprB$, $\Delta sprAB$, and $\Delta sprABD$ on skim milk-containing agar medium. These mutants formed a clear zone of the same size on skim milk-containing medium as the wild-type strain. In Fig. 8, the formation of a clear zone by the wild type and the mutant $\Delta sprABD$ is shown. Therefore, the three chymotrypsins are not involved in the hydrolysis of skim milk. The same was true for the two trypsins whose transcription is under the control of AdpA; mutant $\Delta sprTU$ formed a clear zone of the same size as the wild-type strain. However, mutant $\Delta adpA$ formed a much smaller clear zone than the wild-type strain, indicating that AdpA activated not only these chymotrypsin and trypsin genes but also some other protease genes responsible for the hydrolysis of skim milk. The low extracellular protease activities in mutant $\Delta adpA$ is not due to a defect in protein secretion, because this mutant produced the same extracellular cellulase activity as the wild-type strain when assayed on carboxymethyl cellulose-containing agar medium (data not shown).

DISCUSSION

Control of extracellular proteases by A-factor via AdpA. The present study has demonstrated that AdpA is a master regulator for the three extracellular chymotrypsin-type proteases, because (i) almost no transcription of *sprA*, *sprB*, or *sprD* occurred in mutant $\Delta adpA$, (ii) AdpA bound the upstream activation sites (UASs) in front of their promoters, and (iii) mutations in the UASs severely impaired the transcription of these genes. We previously showed that two extracellular trypsin-type proteases, SprT and SprU (9), and a metalloendopeptidase, SgmA (12), are also under the strict control of AdpA. Since A-factor switches over the cell physiology from the vegetative growth to the differentiation conditions, these proteases begin to be produced at or just before the decision point. The decision point is at the middle of the exponential growth phase (20), at which point A-factor is accumulated in the cell to reach a critical concentration (1). Therefore, these proteases function during the second exponential growth phase after the decision point.

Growth stage-dependent regulation of extracellular proteases, in addition to the A-factor regulation. Although AdpA served as a master switch for the chymotrypsins and trypsins, control of them appeared to be growth phase dependent, in addition to the regulation by the A-factor regulatory cascade. The transcriptional patterns of *sprA* and *sprB* showed an apparent and interesting growth stage-dependent regulation (Fig. 2B); they were actively transcribed at 24 h and at 72 h under the control of AdpA, but the transcription almost completely ceased from 36 h to 60 h. During this gap period when AdpA should be available, some factor or some mechanism may repress the transcription of these genes. Concerning *sprD*, which was transcribed at 24 h but not at 48 h or thereafter, we speculate that some other factor or mechanism that appears after 48 h represses its transcription. These speculative repressors and mechanisms can explain the growth-dependent regulation of *sprA* and *sprB*, although we have no direct evidence for the presence of the repressors.

Similar growth phase-dependent regulation was also observed for *sprT*, *sprU*, and *sgmA*, all of which are controlled by AdpA. The transcription of *sprT* and *sprU* started at 48 h and reached a maximum at 72 h. *sgmA* was transcribed throughout growth, once switched on by AdpA at 24 h, with a rapid and great increase in transcription at 72 h (12). An interesting observation with respect to *sgmA* was that a mutation in one of the two AdpA-binding sites, the A site near the promoter (nucleotide position at about –50, with respect to the transcriptional start point of *sgmA*), completely abolished the *sgmA* transcription, but a mutation in the B site at nucleotide position –260 abolished only the high-level transcription at 72 h. One explanation for this transcriptional pattern is that during the early growth stage before 72 h, some repressor able to bind B site or its neighboring region prevents AdpA from binding to the B site, thus resulting in inhibition of full activation. These findings suggest that once AdpA switches on the transcription of these chymotrypsin and trypsin genes, they are further controlled by unknown factors and mechanisms, perhaps in different ways from one another.

Multiple AdpA-binding sites for transcriptional activation of target genes. For transcriptional activation by AdpA, *sprA*

required two AdpA-binding sites at positions -375 and -50 but *sprD* required only one, at position -40 , of the two AdpA-binding sites. For *sprB*, a single AdpA-binding site at position -50 was sufficient for transcriptional activation by AdpA. We have so far characterized more than 15 genes that are activated by AdpA (24, 35). Some genes required only a single AdpA-binding site located in most cases at -40 to -50 , but others required two AdpA-binding sites, one of which was located in most cases at position -50 and the other of which was located at various positions, for example, at position -375 for *sprA* and at position -200 for *amfR* (34). For both cases, at least one of the AdpA-binding sites was present at positions -40 to -50 in front of the promoter of most target genes. However, for example, *ssgA* required two AdpA-binding sites at positions -240 and -110 (33). How the molecular mechanisms by which AdpA activates the transcription of the target genes and the AdpA molecule bound to the site far from the promoter, in conjunction with the AdpA molecule bound near the promoter, are involved in the transcriptional activation is still unclear. Because AdpA appears to recruit RNA polymerase to the promoter of the target genes and facilitate the isomerization of the RNA polymerase-DNA complex into an open complex competent for transcriptional initiation (30, 35), we can say that the AdpA molecule bound near the promoter interacts with RNA polymerase and controls its transcriptional activity.

Our recent study on the autorepression of *adpA* (11) gives us a hint of the role of the AdpA molecule bound far from the promoter. AdpA appeared to repress its own transcription mainly by cooperative binding to a strong binding site, site 1, at position -110 and a weak binding site, site 2, exactly on the promoter region, thus preventing RNA polymerase from access to the promoter. The AdpA dimer bound to site 1 apparently recruits another AdpA dimer to site 2 and forms a DNA loop via the interaction between the two AdpA dimers. According to this putative model, we assume that in the case of transcriptional activation of target genes by AdpA, an interaction between the AdpA dimer bound at regions far upstream of the promoter and the AdpA dimer bound near the promoter facilitates the transcriptional initiation by RNA polymerase. In the transcriptional activation of *sprD*, AdpA apparently bound at position -40 and facilitated RNA polymerase in forming an open complex formation without any interaction with the AdpA bound at position -180 , as was the case for *sprB*. It is also possible that for some target genes containing two AdpA-binding sites, the AdpA dimer bound far from the promoter recruits another AdpA dimer to position at -40 to -50 so that the recruited AdpA can interact with RNA polymerase. Conceivably, the presence of AdpA on one binding site increases the local concentration of AdpA.

Possible importance of extracellular proteases for morphological development. Most cells in substrate mycelium die during aerial mycelium formation, because the aerial mycelium reuses material first assimilated into the substrate mycelium (19, 31). It is therefore conceivable that many hydrolytic enzymes, such as proteases, nucleases, and lipases, required for the degradation of cytoplasmic contents are produced at a specific time at the beginning of aerial mycelium formation. In addition to this lysis of substrate mycelium, several observations of a possible relationship between extracellular serine proteases and morphological differentiation have been re-

ported. Kim et al. (14) observed that a trypsin inhibitor abolished or impaired aerial mycelium formation in several *Streptomyces* spp. Kim and Hong (17) observed that addition of a serine protease inhibitor, pefabloc SC, to *S. griseus* caused a delay in aerial mycelium formation by 1 to 2 days. Inhibition of aerial mycelium formation by a trypsin inhibitor was also observed by Nicieza et al. (21). Kim and Lee (15, 16) studied a trypsin in *Streptomyces exfoliatus*, in combination with leupeptin and its inactivating enzyme, and concluded that the trypsin was involved in the lysis of substrate hyphae. Ginther (5) and Gibb and Strohl (4) also reported a correlation between a serine protease and morphological differentiation. These observations tempt us to speculate that serine proteases are involved in morphological differentiation in some way. However, even mutant Δ *sprABD* or Δ *sprTU* formed aerial hyphae and spores normally. The chymotrypsins and trypsins under the control of AdpA are all serine proteases and inhibited by serine protease inhibitors. Speculatively, some remaining proteases may compensate the defects in protease activity in these mutants.

In addition to the three chymotrypsins and two trypsins, a protease(s) that hydrolyzed skim milk in the medium was also dependent on AdpA. Our recent study of the *Streptomyces* subtilisin inhibitor (SSI) gene in *S. coelicolor* A3(2) (10) showed that an SSI null mutation greatly increases not only chymotrypsin and trypsin activities but also the skim milk-hydrolyzing activity. Therefore, the protease(s) responsible for the hydrolysis of skim milk is a subtilisin-type serine protease(s) in *S. griseus*, too. The genome sequence of *S. griseus* (our unpublished data) predicts the presence of many proteases, and the number of even predictable subtilisin-type serine proteases is more than 10. Again, it would be difficult for us to identify the proteases responsible for the hydrolysis of skim milk and to determine the role of an individual protease due to the possible mutual compensation of each of the proteases for the others.

Recently, the SSI gene in *S. coelicolor* A3(2) has been found to be controlled by AdpA (13). SSIs modulate the activity of extracellular serine proteases, including subtilisin-type proteases, chymotrypsins, and trypsins, by direct binding (29). We also found that the SSI gene in *S. griseus* is a member of the AdpA regulon (unpublished data). Although disruption of the *S. coelicolor* A3(2) SSI gene gave no morphological changes (10, 13), we are currently studying the role of the SSI in *S. griseus*. Combination of the SSI with its target proteases, including the three chymotrypsins and two trypsins in *S. griseus* that have a genetic background different from that of *S. coelicolor* A3(2) (3), might give us some clue to the role of the proteases in morphological differentiation.

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REFERENCES

1. Ando, N., K. Ueda, and S. Horinouchi. 1997. A *Streptomyces griseus* gene (*sgaA*) suppresses the growth disturbance caused by high osmolality and a high concentration of A-factor during early growth. *Microbiology* **143**:2715-2723.
2. Brown, K. L., S. Wood, and M. J. Buttner. 1992. 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.
3. Chater, K. F., and S. Horinouchi. 2003. Signalling early developmental events in two highly diverged *Streptomyces* species. *Mol. Microbiol.* **48**:9–15.
 4. Gibb, G. D., and W. R. Strohl. 1988. Physiological regulation of protease activity in *Streptomyces peucetius*. *Can. J. Microbiol.* **34**:187–190.
 5. Ginther, C. L. 1979. Sporulation and the production of serine protease and cephamycin C by *Streptomyces lactamdurans*. *Antimicrob. Agents Chemother.* **15**:522–526.
 6. Henderson, G., P. Krygsmann, C. J. Liu, C. C. Davey, and L. T. Malek. 1987. Characterization and structure of genes for proteases A and B from *Streptomyces griseus*. *J. Bacteriol.* **169**:3778–3784.
 7. Horinouchi, S. 2002. A microbial hormone, A-factor, as a master switch for morphological differentiation and secondary metabolism in *Streptomyces griseus*. *Front. Biosci.* **7**:d2045–d2057.
 8. Horinouchi, S., and T. Beppu. 1994. A-factor as a microbial hormone that controls cellular differentiation and secondary metabolism in *Streptomyces griseus*. *Mol. Microbiol.* **12**:859–864.
 9. Kato, J., W.-J. Chi, Y. Ohnishi, S.-K. Hong, and S. Horinouchi. 2005. Transcriptional control by A-factor of two trypsin genes in *Streptomyces griseus*. *J. Bacteriol.* **187**:286–295.
 10. Kato, J., S. Hirano, Y. Ohnishi, and S. Horinouchi. The *Streptomyces* subtilisin inhibitor (SSI) gene in *Streptomyces coelicolor* A3(2). *Biosci. Biotechnol. Biochem.* in press.
 11. Kato, J., Y. Ohnishi, and S. Horinouchi. 2005. Autorepression of AdpA of the AraC/XylS family, a key transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. *J. Mol. Biol.* **350**:12–26.
 12. Kato, J., A. Suzuki, H. Yamazaki, Y. Ohnishi, and S. Horinouchi. 2002. Control by A-factor of a metalloendopeptidase gene involved in aerial mycelium formation in *Streptomyces griseus*. *J. Bacteriol.* **184**:6016–6025.
 13. Kim, D.-W., K. Chater, K.-J. Lee, and A. Hesketh. 2005. Changes in the extracellular proteome caused by the absence of the *bldA* gene product, a developmentally significant tRNA, reveal a new target for the pleiotropic regulator AdpA in *Streptomyces coelicolor*. *J. Bacteriol.* **187**:2957–2966.
 14. Kim, I. S., S. G. Kang, and K. J. Lee. 1995. Physiological importance of trypsin-like protease during morphological differentiation of streptomycetes. *J. Microbiol.* **33**:315–321.
 15. Kim, I. S., and K. J. Lee. 1995. Physiological roles of leupeptin and extracellular proteases in mycelium development of *Streptomyces exfoliatus* SMF13. *Microbiology* **141**:1017–1025.
 16. Kim, I. S., and K. J. Lee. 1996. Trypsin-like protease of *Streptomyces exfoliatus* SMF13, a potential agent in mycelial differentiation. *Microbiology* **142**:1797–1806.
 17. Kim, J.-M., and S.-K. Hong. 2000. *Streptomyces griseus* HH1, an A-factor deficient mutant, produces diminished level of trypsin and increased level of metalloproteases. *J. Microbiol.* **38**:160–168.
 18. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499–560.
 19. Miguélez, E. M., C. Hardisson, and M. B. Manzanal. 1999. Hyphal death during colony development in *Streptomyces antibioticus*: morphological evidence for the existence of a process of cell deletion in a multicellular prokaryote. *J. Cell Biol.* **145**:515–525.
 20. Neumann, T., W. Piepersberg, and J. Distler. 1996. Decision phase regulation of streptomycin production in *Streptomyces griseus*. *Microbiology* **142**:1953–1963.
 21. Niecieza, R. G., J. Huergo, B. A. Connolly, and J. Sanchez. 1999. Purification, characterization, and role of nucleases and serine proteases in *Streptomyces* differentiation. Analogies with the biochemical processes described in late steps of eukaryotic apoptosis. *J. Biol. Chem.* **274**:20366–20375.
 22. Ohnishi, Y., and S. Horinouchi. 2004. The A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces*. *Biofilms* **1**:319–328.
 23. Ohnishi, Y., S. Kameyama, H. Onaka, and S. Horinouchi. 1999. The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. *Mol. Microbiol.* **34**:102–111.
 24. Ohnishi, Y., H. Yamazaki, J. Kato, A. Tomono, and S. Horinouchi. 2005. AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*. *Biosci. Biotechnol. Biochem.* **69**:431–439.
 25. Sidhu, S. S., G. B. Kalmar, and T. J. Borgford. 1993. Characterization of the gene encoding the glutamic-acid-specific protease of *Streptomyces griseus*. *Biochem. Cell Biol.* **71**:454–461.
 26. Sidhu, S. S., G. B. Kalmar, L. G. Willis, and T. J. Borgford. 1994. *Streptomyces griseus* protease C. A novel enzyme of the chymotrypsin superfamily. *J. Biol. Chem.* **269**:20167–20171.
 27. Sidhu, S. S., G. B. Kalmar, L. G. Willis, and T. J. Borgford. 1995. Protease evolution in *Streptomyces griseus*. Discovery of a novel dimeric enzyme. *J. Biol. Chem.* **270**:7594–7600.
 28. Strohl, W. R. 1992. Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucleic Acids Res.* **20**:961–974.
 29. Taguchi, S., S. Kojima, M. Terabe, K. Miura, and H. Momose. 1994. Comparative studies on the primary structures and inhibitory properties of subtilisin-trypsin inhibitors from *Streptomyces*. *Eur. J. Biochem.* **220**:911–918.
 30. Tomono, A., Y. Tsai, H. Yamazaki, Y. Ohnishi, and S. Horinouchi. 2005. Transcriptional control by A-factor of *strR*, the pathway-specific transcriptional activator for streptomycin biosynthesis in *Streptomyces griseus*. *J. Bacteriol.* **187**:5595–5604.
 31. Wildermuth, H. 1970. Development and organization of the aerial mycelium in *Streptomyces coelicolor*. *J. Gen. Microbiol.* **60**:43–50.
 32. Yamazaki, H., Y. Ohnishi, and S. Horinouchi. 2000. An A-factor-dependent extracytoplasmic function sigma factor (σ^{AdfA}) that is essential for morphological development in *Streptomyces griseus*. *J. Bacteriol.* **182**:4596–4605.
 33. Yamazaki, H., Y. Ohnishi, and S. Horinouchi. 2003. Transcriptional switch on of *sgsA* by A-factor, which is essential for spore septum formation in *Streptomyces griseus*. *J. Bacteriol.* **185**:1273–1283.
 34. Yamazaki, H., Y. Takano, Y. Ohnishi, and S. Horinouchi. 2003. *amfR*, an essential gene for aerial mycelium formation, is a member of the AdpA regulon in the A-factor regulatory cascade in *Streptomyces griseus*. *Mol. Microbiol.* **50**:1173–1187.
 35. Yamazaki, H., A. Tomono, Y. Ohnishi, and S. Horinouchi. 2004. DNA-binding specificity of AdpA, a transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*. *Mol. Microbiol.* **53**:555–572.
 36. Yoshida, N., S. Tsuruyama, K. Nagata, K. Hirayama, K. Noda, and S. Makisumi. 1988. Purification and characterization of an acidic amino acid specific endopeptidase of *Streptomyces griseus* obtained from a commercial preparation (Pronase). *J. Biochem.* **104**:451–456.