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Characterization of an A-Factor-Responsive Repressor for *amfR* Essential for Onset of Aerial Mycelium Formation in *Streptomyces griseus*

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A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) is essential for the initiation of aerial mycelium formation in *Streptomyces griseus*. *amfR* is one of the genes which, when cloned on a low-copy-number plasmid, suppresses the aerial mycelium-negative phenotype of an A-factor-deficient mutant of *S. griseus*. Disruption of the chromosomal *amfR* gene resulted in complete abolition of aerial mycelium formation, indicating that *amfR* is essential for the onset of morphogenesis. Cloning and nucleotide sequencing of the region upstream of *amfR* predicted an operon consisting of *orf5*, *orf4*, and *amfR*. Consistent with this idea, Northern blotting and S1 mapping analyses suggested that these three genes were cotranscribed mainly by a promoter (P_{ORF5}) in front of *orf5*. Furthermore, P_{ORF5} was active only in the presence of A-factor, indicating that it is A-factor dependent. Gel mobility shift assays showed the presence of a protein (AdpB) able to bind P_{ORF5} in the cell extract from an A-factor-deficient mutant but not from the wild-type strain. AdpB was purified to homogeneity and found to bind specifically to the region from −72 to −44 bp with respect to the transcriptional start point. Runoff transcriptional analysis of P_{ORF5} with purified AdpB and an RNA polymerase complex isolated from vegetative mycelium showed that AdpB repressed the transcription in a concentration-dependent manner. It is thus apparent that AmfR as a switch for aerial mycelium formation and AdpB as a repressor for *amfR* are members in the A-factor regulatory cascade, leading to morphogenesis.

The gram-positive bacterial genus *Streptomyces* shows characteristic morphological differentiation, which is a useful target for the genetic analysis of procaryotic multicellular differentiation (5–7). On solid media, *Streptomyces* spp. form branched, multinucleoid substrate hyphae during vegetative growth. In response to nutritional limitation, older parts of the substrate hyphae produce aerial mycelium. After septa have been formed at regular intervals along the hyphae, long chains of uninucleoid spores are formed.

Our approach to studying morphological differentiation in *Streptomyces* spp. has been focused on a hormonal substance, A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone), which is essential for the initiation of aerial mycelium formation and streptomycin production in *Streptomyces griseus* (9, 18). An A-factor-deficient mutant of *S. griseus* shows a bald (Bld) phenotype, deficient in both aerial mycelium and spore formation, and exogenous supply of A-factor at an extremely low concentration to this strain induces aerial mycelium and spore formation (13, 14). Taking advantage of the Bld phenotype caused by A-factor deficiency, we have cloned genes that cause aerial mycelium and spore formation by using this mutant strain as a host (12). This strategy brought forth three sets of genes, *amfR*, *amfA*, and *amfB* (30); *orf1590* (1); and *amfC* (20). Among these, *orf1590* was also shown by Babcock and Kendrick (1, 2) to complement a Bld phenotype of one class of mutants of *S. griseus*. *amfR* encodes a protein similar to the response regulators of two-component regulatory systems, and the *amfA* and *amfB* products are members of a family of bacterial membrane translocators. Ma and Kendall (21) cloned an *amfRAB* homologous locus, named *ram*, from *Streptomyces coelicolor* A3(2), as a gene cluster that caused accelerated aerial mycelium formation in *Streptomyces lividans*.

In this study we show that *amfR* is essential for aerial growth. Identification of a protein (AdpB) able to bind the promoter of the *amfR* operon only in the absence of A-factor and repressor-like behavior of AdpB observed in runoff transcription experiments strongly suggest that AmfR and AdpB are important members in the A-factor regulatory cascade, leading to aerial mycelium formation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. griseus* IFO 13350, obtained from the Institute of Fermentation, Osaka, Japan, was the parental strain of an A-factor-deficient mutant strain, HH1 (16). This mutant strain, obtained by incubation at 37°C, was defective in aerial mycelium formation and streptomycin production because of its A-factor deficiency. Plasmid pIJ486 (carrying thiostrepton resistance) (32) has a copy number of 40 to 100 per genome. Plasmids pIJ922 (11) and pTMA1 (31) (both carrying thiostrepton resistance) have a copy number of 1 to 2 per genome, as judged by agarose gel electrophoresis (30). pKU206 (carrying thiostrepton resistance), an SCP2*-derived plasmid, was obtained from H. Ikeda (17). DNA was manipulated in *Escherichia coli* JM109 [Δ(*lac-pro*) *thi-1 endA1 gyrA96 hsdR17 relA1 recA1*/F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*] (33) by cloning in pUC18 and pUC19 (33). *S. griseus* strains were grown in Bennett-glucose medium (containing the following [grams per liter]): yeast extract [Difco Laboratory], 1; meat extract [Kyokuto Co.], 1; N. Z. amine [Wako Pure Chemical], 2; and glucose, 10, (pH 7.2), YMPG medium (9), and nutrient agar medium (Difco Laboratory). Growth conditions for *E. coli* strains were as described by Maniatis et al. (22).

General recombinant DNA techniques. Restriction endonucleases and other DNA-modifying enzymes were purchased from Takara Shuzo Co., Kyoto, Japan.

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[α-^{32}P]dCTP at 110 TBq/mmol for nucleotide sequencing by the M13-dideoxynucleotide method with M13mp18 and M13mp19 (33) and for the Takara multiprime DNA labeling system and [γ-^{32}P]ATP at >220 TBq/mmol for 5′-end labeling of probe DNA for primer extension and gel mobility shift assays were purchased from Amersham International. Thiostrepton was a gift from Asahi Chemical Industry, Shizuoka, Japan. DNA manipulations in *E. coli*, Southern hybridization, and colony hybridization were as described by Maniatis et al. (22), and those in *Streptomyces* spp. were as described by Hopwood et al. (11).

Cloning and subcloning of the region upstream from *amfR*. The previously cloned 9-kb *Sau*3AI fragment on pSPO1 was inserted at the *Bam*HI site of pIJ487 (30). The *Sma*I site of pUC19 was first changed into an *Nco*I site by use of an 8-mer *Nco*I linker (pUC-Nco), and the 1.6-kb *Bam*HI-*Nco*I fragment was cloned between the *Bam*HI and *Nco*I sites of pUC-Nco to generate plasmid pUC-A. The 1.6-kb *Bam*HI-*Nco*I fragment from pUC-A was used as a ^{32}P-labeled hybridization probe, and an approximately 6.5-kb *Nco*I fragment giving a positive signal by Southern hybridization against the chromosomal DNA from *S. griseus* IFO 13350 was detected. The 6.5-kb *Nco*I fragment was cloned by colony hybridization at the *Nco*I site of pUC-Nco to generate pUC-F. The nucleotide sequence of the 2-kb region upstream from *amfR* was determined and combined with the previous sequence data.

The 3.5-kb *Eco*RI-*Nco*I fragment containing *orf5*, *orf4*, and *amfR* (the *orf5-orf4-amfR* operon) (see Fig. 1) was recovered as an *Eco*RI fragment from pUC-F and cloned at the *Eco*RI site of pTMA1 to generate pAFL1. For construction of pAFL1Δ, the *Eco*RI fragment from pUC-F was first cloned onto pUC19. This plasmid was cleaved by *Pma*CI, and 8-mer *Bgl*II linkers were attached to the ends. After digestion with *Bgl*II, it was religated to form a circular DNA. The DNA fragment with an internal deletion of the 384-bp *Pma*CI fragment was recovered as an *Eco*RI fragment and finally cloned onto pTMA1. For construction of pAFL2, the 3.5-kb *Eco*RI fragment from pUC-F was partially digested with *Eco*47III and the 1.9-kb *Eco*RI-*Eco*47III fragment was cloned between the *Eco*RI and *Sma*I sites of pUC19. The fragment was recovered as an *Eco*RI-*Hin*dIII fragment and inserted into pTMA1. For construction of pSL6, the 1.6-kb *Bam*HI-*Nco*I fragment was recovered as a *Bam*HI-*Eco*RI fragment from pUC-A and inserted between the *Bam*HI and *Eco*RI sites of pTMA1. For construction of pSH11 and pAFL11, the 316-bp *Eco*RI-*Eco*47III fragment carrying the promoter region in front of ORF5 was first cloned between the *Eco*RI and *Sma*I sites of pUC19, then recovered as an *Eco*RI-*Hin*dIII fragment, and inserted into pIJ487 and pTMA1, respectively.

The transformants were viewed microscopically as well as macroscopically to determine whether they produced aerial mycelium and spores.

Gene disruption. The chromosomal *amfR* gene was disrupted according to the procedure used for *amfC* disruption (19). The 1.6-kb *Bam*HI-*Nco*I region carrying the entire *amfR* gene was first cloned onto pUC19 as a *Bam*HI fragment after the *Nco*I site had been changed into a *Bam*HI site with an 8-mer *Bam*HI linker. The unique *Eco*47III site in the middle of the *amfR*-coding region was changed into a *Bgl*II site with an 8-mer *Bgl*II linker, and the 1.7-kb *Bam*HI fragment containing the kanamycin resistance (*aphII*) gene (3) was inserted into the newly created *Bgl*II cleavage site to construct a mutagenized *amfR* gene. The mutagenized *amfR* sequence with *aphII* was excised with *Bam*HI as a 3.3-kb fragment and ligated into the *Bam*HI site in pKU206 to construct plasmid pRD1. pKU206 is known to be unstable in *S. griseus* in the absence of thiostrepton used as a selection marker. pRD1 was introduced by transformation into the wild-type strain, *S. griseus* IFO 13350. Thiostrepton (20 μg/ml)- and kanamycin (20 μg/ml)-resistant transformants were then cultured at 30°C for 72 h in YMPG liquid medium without thiostrepton and plated onto YMPG agar plates containing 20 μg of kanamycin per ml. Kanamycin-resistant colonies thus obtained were then checked for their sensitivity to thiostrepton, and finally four colonies showing thiostrepton sensitivity and kanamycin resistance were obtained. All four colonies showed a Bld phenotype on YMPG agar plates, and correct disruption of *amfR* in one of the colonies was confirmed by Southern blot hybridization with the 1.6-kb *Bam*HI-*Nco*I fragment carrying the intact *amfR* gene and the 1.7-kb *Bam*HI fragment containing *aphII* as probes.

Northern blot analysis. *S. griseus* IFO 13350 and HH1 were grown at 30°C for 24 to 60 h in YMPG liquid medium. Strain HH1 was grown with or without exogenous A-factor at a final concentration of 50 ng/ml. Total cellular RNA was isolated as described previously (15) and quantified by the absorbance at 260 nm. Northern hybridization was carried out by the formaldehyde method according to Maniatis et al. (22). Fifteen micrograms of RNA was loaded onto each lane, electrophoresed on a 1% agarose gel, and transferred to a Hybond-N$^+$ membrane (Amersham) according to the manufacturer's recommendation. Hybridization was performed at 65°C for 18 h, and the blot was washed three times with 2× SSC (1× SSC contains 0.15 M sodium citrate and 0.15 M NaCl, pH 8.0) containing 0.1% sodium dodecyl sulfate (SDS) at 65°C for 10 min. The probes were labeled with [α-^{32}P]dCTP by the *Bca*BEST random primer DNA labelling kit (Takara Shuzo) according to the protocol supplied. The probes were an 890-bp fragment containing the entire *orf5* sequence and a 613-bp fragment containing the *amfR*-coding sequence, both of which were amplified by the standard PCR method with the following oligonucleotide primers (see Fig. 1): 5′-GCCGAATTCGGAACAACGATGTGC (5N) and 5′-CGGCTCGAGCGCC GAGCGGTAGTGG (5C) for *orf5* and 5′-GCCGAATTCGACTACCGTGC TGC (RN) and 5′-CGGCTCGAGGATCCAGCCCGCTTCC (RC) for *amfR*.

S1 mapping. The 320-bp *Eco*RI-*Eco*47III fragment (see Fig. 1) with ^{32}P at the *Eco*47III end was prepared by standard DNA manipulation and used as a hybridization probe. For clearer resolution of the end of the protected fragment, another ^{32}P-labeled fragment used as a probe was prepared as follows. An 18-mer oligonucleotide, 5′-GATTCCCCGCTCTTTCGT-3′ (nucleotide positions +106 to +89), was labeled with [γ-^{32}P]ATP with T4 polynucleotide kinase. For preparation of a ^{32}P-labeled 130-bp probe, PCR was carried out with this oligonucleotide and a nonlabeled 21-mer oligonucleotide, 5′-GTGGAATTCAC CATCGGCGGA-3′ (nucleotide positions −75 to −55), under standard conditions. RNA was prepared from *S. griseus* IFO 13350 and strain HH1 grown at 30°C for 2 days in YMPG medium. RNA (100 μg) was hybridized with 100,000 Cerenkov cpm of the probe, as described previously (15). Maxam-Gilbert sequencing ladders on a 12% polyacrylamide sequencing gel (23) were generated with the same ^{32}P-labeled fragment.

Fractionation of cell extracts by DEAE-Toyopearl column chromatography for gel mobility shift assays. *S. griseus* HH1 was cultured in 100 ml of YMPG medium at 30°C for 48 h. The mycelium was homogenized with a glass homogenizer and transferred to 1 liter of YMPG medium in a 5-liter flask. After cultivation at 30°C for 48 h, the mycelium was harvested by centrifugation. The mycelium was suspended in 100 ml of buffer A (containing 10 mM Tris-HCl [pH 7.0], 1 mM EDTA, 1 mM dithiothreitol, 10% [vol/vol] glycerol) and disrupted by sonication. The supernatant obtained by centrifugation of the disrupted mycelium at 15,000 × *g* for 30 min at 4°C was applied to a DEAE-Toyopearl open column (4.6 by 15 cm; Tosoh Corp., Tokyo, Japan) previously equilibrated with buffer A. After the column had been washed with 750 ml of buffer A, proteins were eluted with a linear KCl gradient of 0 to 1 M in a total volume of 200 ml at a flow rate of 2.5 ml/min. The cell extract from strain HH1 grown in the presence of 50 ng of A-factor per ml was similarly fractionated.

Gel mobility shift assay. DNA-binding determinations by mobility shift assays were done essentially by the method of Chodosh (8). For the standard binding assay, 0.5 to 5 ng of ^{32}P-labeled double-stranded DNA (10,000 to 20,000 cpm) was incubated with 5 to 20 μg of proteins at 30°C for 30 min in binding buffer containing 10 mM Tris-HCl (pH 7.0), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (vol/vol) glycerol, 1 μg of poly(dI-dC)-poly(dI-dC), and 50 μg of bovine serum albumin per ml in a total volume of 50 μl. After incubation, complexes and free DNA were resolved on nondenaturing polyacrylamide gel containing 6% acrylamide and 0.075% bisacrylamide with running buffer containing 40 mM Tris-HCl (pH 8.0), 20 mM sodium acetate, and 1 mM EDTA. Gels were dried and subjected to autoradiography with a Du Pont Cronex intensifying screen.

Purification of the DNA-binding protein. *S. griseus* HH1 was used as a source of the DNA-binding protein. SDS-polyacrylamide gel electrophoresis (PAGE) on slab gels was used to monitor protein purification and to estimate molecular sizes under denaturing conditions. The concentrations of polyacrylamide were 12.5% in separating gels and 4% in stacking gels. The gels were stained with 0.1% Coomassie brilliant blue R-250. During purification, protein concentrations were measured with a Bio-Rad protein assay kit with bovine serum albumin as the standard.

(i) Preparation of cell extract. *S. griseus* HH1 was cultured in 100 ml of YMPG medium at 30°C for 48 h. The mycelium was homogenized with a glass homogenizer and transferred to 15 liters of the same medium in a 30-liter jar fermentor. After cultivation at 30°C for 48 h, the mycelium was harvested by centrifugation. Mycelium (wet weight, 300 g) was suspended in 1 liter of buffer A containing 10 mM Tris-HCl (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol, and disrupted by three passages at 700 kg/cm^2 through a Manton-Gaulin apparatus (model 15M8TA; Gaurin Corp., Everett, Mass.). The supernatant, obtained by centrifugation of the disrupted mycelium at 10,000 × *g* for 30 min at 4°C, was used as the cell extract. Proteins (10.2 g) were recovered through this step.

(ii) Ammonium sulfate fractionation. Solid ammonium sulfate was added to the cell extract to 30% (wt/vol) saturation, and the mixture was gently stirred at 4°C overnight. The precipitate was obtained by centrifugation at 15,000 × *g* for 30 min, dissolved in 200 ml of buffer A, and dialyzed overnight against buffer A. Proteins (952 mg) were recovered through this step.

(iii) DEAE-Toyopearl column chromatography. The dialyzed sample was applied to a DEAE-Toyopearl column (3.0 by 40 cm in diameter; Tosoh Corp.) previously equilibrated with buffer A. After the column had been washed with 1 liter of buffer A, proteins were eluted with a linear gradient of KCl from 0 to 1 M in a total volume of 3 liters of buffer A at a flow rate of 2.5 ml/min. Fractions (250 ml) containing DNA-binding activity were pooled and dialyzed overnight against buffer A. Proteins (99 mg) were recovered through this step.

(iv) MonoQ column chromatography. The dialyzed sample was divided into five portions, each of which was applied to a MonoQ HR 10/10 fast protein liquid chromatography column (Pharmacia) equilibrated with buffer A, because of the small capacity of the column. Proteins were eluted with a linear gradient of 0 to 1 M KCl in buffer A in a total volume of 100 ml at a flow rate of 1 ml/min. One cycle of chromatography gave fractions (30 ml each) containing activity. These fractions, obtained from five cycles of chromatography, were collected and dialyzed overnight against buffer A. Proteins (31 mg) were recovered through this step.

(v) Heparin affinity column chromatography. The dialyzed sample was divided into three portions, each of which was applied to a HiTrap heparin fast protein

liquid chromatography column (Pharmacia) equilibrated with buffer A. Proteins were eluted with a linear gradient of 0 to 1 M KCl in buffer A in a total volume of 100 ml at a flow rate of 0.5 ml/min. One cycle of chromatography gave fractions (5 ml each) containing activity. These fractions, obtained from three cycles of chromatography, were collected and dialyzed overnight against buffer A. Proteins (5 mg) were recovered through this step.

(vi) Nondenaturing PAGE and protein elution. The above-mentioned sample was concentrated to 2 ml by membrane filtration and separated by nondenaturing PAGE (native PAGE; containing 10 and 2.5% polyacrylamide in the separating and stacking gels, respectively). After identification of the DNA-binding protein by Southwestern blot analysis (see below), a gel piece containing the DNA-binding protein was cut out from each of several gels. The gel pieces were then homogenized with a Teflon homogenizer and suspended in 10 ml of buffer A. The suspension was gently stirred at 4°C for 3 h and centrifuged at 10,000 × *g* for 20 min to remove gel particles. This elution procedure was repeated three times. The eluates were collected and concentrated by membrane filtration to yield 1 ml of the purified protein solution. The amount of AdpB recovered was 1.2 mg.

Southwestern blotting. Southwestern blotting assay was done essentially by the method of Miskimins et al. (24). The fraction (4 μg of protein) eluted from the heparin affinity column was divided in two and separated by native PAGE, described above. One lane was stained with Coomassie brilliant blue, and the proteins in the other lane were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was incubated at 30°C for 1 h with 0.1 μg of a ^{32}P-labeled 314-bp *Eco*RI-*Eco*47III fragment in 20 ml of the binding buffer used for the gel mobility shift assay. The membrane was washed with the binding buffer containing 0.2 M KCl and subjected to autoradiography with a Du Pont Cronex intensifying screen.

Generation of trimmed fragments for retardation probes. For generation of a deletion series from the 314-bp *Eco*RI-*Eco*47III fragment, the original 314-bp fragment was first cloned into pUC19 between the *Eco*RI and *Hin*cII sites and then cleaved with *Eco*RI. The *Eco*RI ends were digested with exonuclease III at 37°C for 10 to 60 s. The exonuclease III-treated fragments were blunt ended with mung bean nuclease and Klenow fragment, and an 8-mer *Eco*RI linker was attached to both ends. After *Eco*RI digestion, they were religated to form circular DNAs. Plasmids containing fragments with appropriate sizes were screened for by agarose gel electrophoresis, and the terminal ends of deletions were determined by nucleotide sequencing. The *Eco*RI-*Sau*3AI fragment was recovered from each of the plasmids, and both ends were ^{32}P labeled with T4 polynucleotide kinase for gel retardation assays.

Run off transcription. The in vitro transcriptional analysis was done according to the procedure described previously (28). An RNA polymerase complex mainly with σ^B promoter specificity, prepared from exponentially growing *S. griseus* cells (28), was used. The *Eco*RI-*Eco*47III and *Eco*RI-*Ava*II fragments (see Fig. 7) were purified by agarose gel electrophoresis and used as the templates. A reaction mixture containing the RNA polymerase complex, the DNA template (0.5 pmol), [α-^{32}P]UTP, nucleoside triphosphate mixture, heparin, and various amounts of AdpB was incubated at 30°C for 30 min to allow a single round of transcription. The transcripts were separated by 8 M urea-PAGE (28). The transcription products were detected by autoradiography. The *Eco*RI-*Eco*47III and *Eco*RI-*Ava*II fragments were expected to yield 244- and 177-bp runoff transcripts, respectively.

Nucleotide sequence accession number. The newly edited sequence of the 2-kb region upstream from *amfR* combined with previous sequence data has been registered in the DDBJ, EMBL, and GenBank databases under accession no. AB006206.

RESULTS

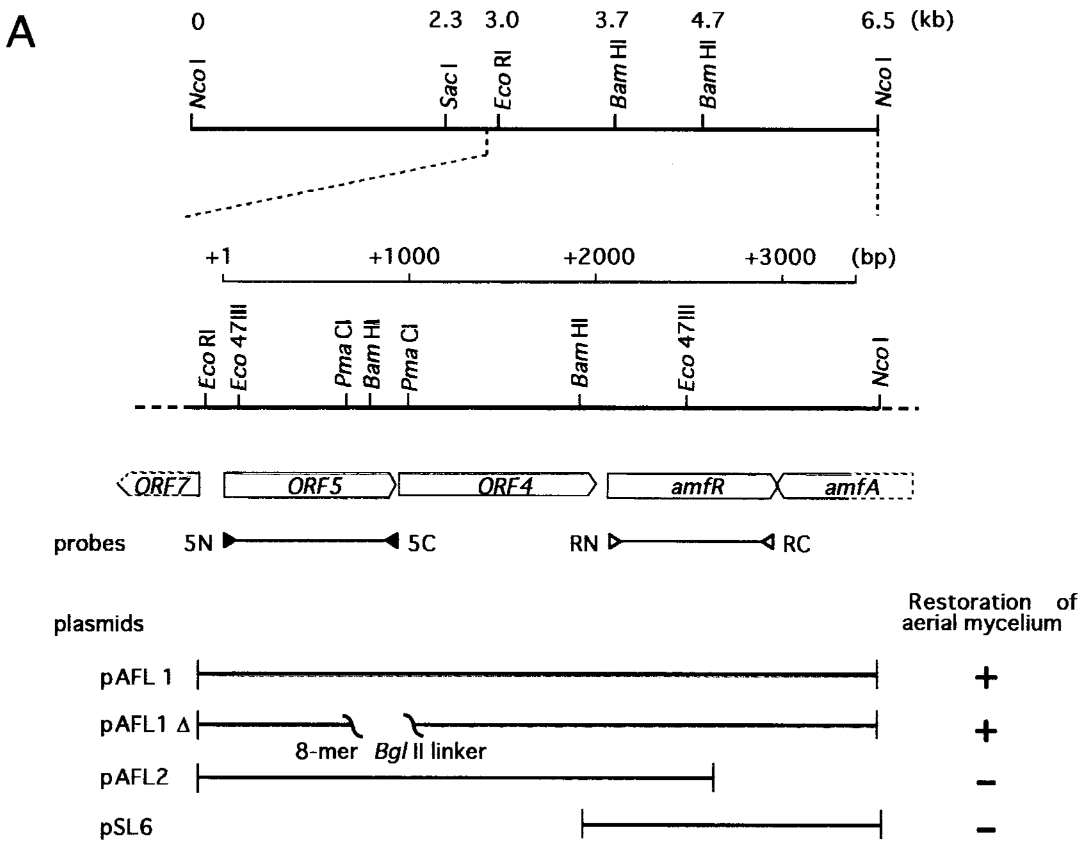
Cloning and nucleotide sequencing of a region upstream from *amfR*. In order to clone the region upstream from *amfR*, we used the 1.6-kb *Nco*I-*Bam*HI fragment containing the whole coding sequence of *amfR* (Fig. 1) as the ^{32}P-labeled hybridization probe against *Nco*I-digested chromosomal DNA of *S. griseus* and detected a signal of 6.5 kb. The 6.5-kb *Nco*I fragment was cloned into pUC-Nco by the standard DNA manipulation, including colony hybridization. The restriction map of the cloned 6.5-kb fragment is shown in Fig. 1.

We determined the nucleotide sequence of the approximately 2-kb region upstream from *amfR*. Computer-aided FRAME analysis (4) on this region predicted two complete open reading frames (ORFs) (ORF4, with 319 amino acids, and ORF5, with 289 amino acids) in the same direction as AmfR and a truncated ORF (ORF7) in the opposite direction to that of AmfR (Fig. 1). In front of *amfR*, *orf5*, and *orf4*, possible ribosome-binding sequences, GAGGG, GGAA, and GGA, respectively, are present at appropriate positions. ORF7 is also preceded by a sequence, GGAGGAA. Neither ORF4 nor ORF5 showed any homology with proteins registered in the Swiss-Prot data bank. On the other hand, the truncated ORF7 represents the 7Fe ferredoxin of *S. griseus* (29), because the 56 amino acids of ORF7 deduced from the nucleotide sequence are identical to the NH_2-terminal portion of the ferredoxin, consisting of 105 amino acids.

Disruption of the chromosomal *amfR* gene. Whether *amfR* was essentially required for aerial mycelium formation had been unclear, since *amfR* was cloned as a suppressor for the aerial mycelium-negative phenotype of the A-factor-deficient mutant strain *S. griseus* HH1 (30). We disrupted the chromosomal *amfR* gene by means of double crossover, with the kanamycin resistance (*aphII*) gene as a selection marker to determine its role in morphogenesis. The insertion of *aphII* by this strategy does not interfere with transcription and translation of *orf4* and *orf5*, because *amfR* is the third gene in this operon. Southern hybridization with the *aphII* sequence as a probe against the *Bam*HI-digested and *Bam*HI-plus-*Hin*dIII-digested chromosomal DNAs identified four true disruptants. All four disruptants showed a Bld phenotype (Fig. 2). Introduction of pAFL1 carrying *amfR* and its upstream region on a low-copy-number plasmid restored sporulation to the same level as that of the wild-type strain. It is therefore concluded that *amfR* plays an essential role in aerial mycelium and spore formation.

Phenotypes conferred on *S. griseus* HH1 by *amfR* together with its upstream region. Our previous study showed that both *amfR* and *amfA* on low-copy-number plasmid pIJ922 were required for aerial mycelium formation in *S. griseus* HH1 as much as for that in the wild-type strain (30). The two genes on pIJ922, with its copy number of 1 to 2, caused abundant aerial mycelium formation, whereas those on high-copy-number plasmid pIJ487, with its copy number of 40 to 100, appeared to inhibit the growth of substrate mycelium, thereby causing less-abundant aerial mycelium formation. The inhibition of growth of substrate mycelium was ascribed as most likely to be caused by overexpression of AmfR (30). Since ORF4 and ORF5, located adjacent to AmfR in the same direction, had been thought to have some functional relationship with AmfR, we wanted to determine whether a region upstream of *amfR* affected aerial mycelium formation in strain HH1. An unexpected finding, as described below, was that only *amfR*, when introduced together with a presumptive promoter region in front of *orf5*, was sufficient to cause aerial mycelium and spore formation in strain HH1.

We performed subcloning experiments as shown at the bottom of Fig. 1A. The 1.6-kb *Bam*HI-*Nco*I fragment containing *amfR* (plasmid pSL6) on low-copy-number plasmid pTMA1 was unable to restore aerial mycelium formation in *S. griseus* HH1. However, the 3.3-kb *Eco*RI-*Nco*I fragment containing *orf5-orf4-amfR*, but not *amfA*, on pTMA1 (plasmid pAFL1) induced aerial mycelium and spore formation in strain HH1 just as abundantly as in the wild-type strain. Plasmid pAFL1Δ, in which *orf5* and *orf4* were disrupted by deletion of the indicated 384-bp *Pma*CI fragment from pAFL1, also restored aerial mycelium and spore formation to the same extent, indicating that neither ORF5 nor ORF4 was required for the restoration. In this plasmid, the insertion of an 8-mer *Bgl*II linker changes the reading frame of ORF4 when ORF4 is translated as an ORF5-ORF4 fusion protein from the initiation codon of *orf5*. In addition, pAFL2, containing *orf5* and *orf4* but not *amfR*, failed to restore aerial mycelium formation. These results indicated that *amfR* together with the region upstream of *orf5* was able to restore sporulation in *S. griseus* HH1 when it was cloned onto the low-copy-number plasmid. The upstream region responsible for aerial mycelium forma-



B

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ATGCAGGCCTTGTCCTTCACGTCGACACAAGGCTGCGCGATGACGTAGGTCACGCTGTCGTTCCTCCTCGGTAGGGCGTTGGCTCTGCCGCGGGAGCGCG  -146
 I  C  A  K  D  K  V  D  V  C  P  Q  A  I  V  Y  T  M (←ORF7)
                                                                                  EcoRI
GCGTCGTCGATGCCCGCCTCTAGTATCTCCGTTCTTGGGCACGATCCGAACAGGAGGGGCGGACAGAGCTGTGGAATTCACCATCGGCGGACGGCTGGAA   -46
                                                                                 |-> 1
 MluI
GTCAGCATTACCCCCGCTGACGTGGGCAAACGCGTGTCCGTTCGCCGCCGGACGGAGAGCGGTGGCACGGGCGCGCAGTTCACCGACGTCGTCGGGGTGC   +54
 |-> 2   |-> 3     |-> 4                         +1
                              Sau3AI
TCACATCATGGAACAACGATGTGCTCTCGATCACACGAAAGAGCGGGGAATCCGTCCACATCGTGGAATCCTCCTTGGTGGCGGGCAAGGTCGTTCCCGC  +154
         (ORF5→) M  C  S  R  S  H  E  R  A  G  N  P  S  T  S  W  N  P  P  W  W  R  A  R  S  F  P  P
                     AvaII                                                               Eco47III
CGCCCCGGCCCGCCGCCGAGGTCCCGCCGCCTCCTTCGGGGAACTGGCCGTCGTCACCGCGCGCGCCTGGCAGCCCGTGGAGAGCGAAGCGCTGGGCGAC  +254
  P  R  P  A  A  E  V  P  P  P  P  S  G  N  W  P  S  S  P  R  A  P  G  S  P  W  R  A  K  R  W  A  T

TGGCGGCTGCGCGCCGCCGGGGGCTTCACCCGGCGCGCCAACTCCGGCGCTGCCGCTCGGCGATCCGGGGCTGCCGGTCGGCGAGGCGCTCGACCGGGTC  +354
 G  G  C  A  P  P  G  A  S  P  G  A  P  T  P  A  L  P  L  G  D  P  G  L  P  V  G  E  A  L  D  R  V

                                                  . . . . . .

GCCTCGGCGGCCTGGCTCCAGGTCGAGGAGGACAACGCGGGGGCGCGGGCACTCTACGACGGCATGGGCTTCGCGGCCCACCACCGCTACCACCACTACC  +934
A  S  A  A  W  L  Q  V  E  E  D  N  A  G  A  R  A  L  Y  D  G  M  G  F  A  A  H  H  R  Y  H  H  Y  R

GCTCGGCGTGACGGACGCCGGTGGCTCCGCGCCGGGCCCTCCGGCGGATCGACGCACGTGAGCGGGGACAGGAACATGGGTCGCATATGAGCGCGCTGGA +1034
  S  A  *  (ORF4→) M  A  P  R  R  A  L  R  R  I  D  A  R  E  R  G  Q  E  H  G  S  H  M  S  A  L  D

                                                  . . . . . .

GGTGACTTCCTGCGCGGGGCCGCGGAGATGGAGGAGTATGCGGCGGTCCTCGACGGCATCGAACCGACGACGGCCGAGAACGTCCGGCGCAAGGCTCACG +1894
G  D  F  L  R  G  A  A  E  M  E  E  Y  A  A  V  L  D  G  I  E  P  T  T  A  E  N  V  R  R  K  A  H  A

CCGCGCGCGCCCTGCTGAACTGAACCGGCCGTCCTGCGGGATCGCGGCCGGAATCCACCCCGTCGACGCCCGCTGCCGCCCTGCCGGTGCCCAGGGAGTG +1994
  A  R  A  L  L  N  *

GCGAGACGGATGGGTCCGTGAGCGATGTGCGCTCCCGATTTACATCCGAACGCCCCCTTCCGCGCCCAACGGGAGCGGTATTCGGCCCGTTTGTCGGCGT +2094

ACGCGAATGTCCATGCGCTCAACTGGATACCCGTCACCGGATGCGTACAGCCCGTCGCCTTCCGCGTCGGGCGGGCCGGAAGAGAAAAGCTGAGGGCAGA +2194
                                                                                           (amfR→)
CTGATGACTACCGTGCTGCTGGTCCACACCGTGCCGTTGTGGCGCGCGTCGCTGGCGTCGCTCCTCGGCACGGGGCGGGGCATAGAGCTCCGCACCGCCG +2294
   M  T  T  V  L  L  V  H  T  V  P  L  W  R  A  S  L  A  S  L  L  G  T  G  R  G  I  E  L  R  T  A  E
```

tion in combination with *amfR* was most likely a presumptive promoter region in front of *orf5* (P_{ORF5}), because the ORF5- and ORF4-coding sequences caused no detectable effect on the restoration of aerial mycelium formation. Introduction of the *Eco*RI-*Eco*47III region in front of *orf5* alone either on the multicopy-number (pSH11) or the low-copy-number (pAFL11) plasmid did not restore sporulation in strain HH1 (data not shown).

Our repeated attempts to clone the 3.3-kb *Eco*RI-*Nco*I fragment onto the high-copy-number plasmid pIJ487 failed, probably because of a deleterious effect of an excess of AmfR due to the gene dosage effect of *amfR*, as was observed previously (30). As described below, strong promoter activity of P_{ORF5} was detected.

Northern blot analysis. The essential involvement of *amfR* in morphogenesis prompted us to determine the transcription of *amfR* in response to A-factor by Northern blot analysis. Total RNAs isolated from the A-factor-deficient mutant of *S. griseus*, strain HH1, cultivated with or without exogenously supplemented A-factor, were hybridized with probe DNA carrying the *amfR*-coding sequence. The mRNA isolated from strain HH1 grown in the presence of A-factor yielded a hybridization signal of 3.5 kb, whereas no signal was detected in the mRNA isolated from strain HH1 grown in the absence of A-factor (Fig. 3A). These findings suggested that *amfR* was transcribed in response to A-factor by a promoter located further upstream of its coding region. The gene organization upstream and downstream of *amfR* (Fig. 1) predicted the presence of a promoter in front of *orf5*. The presence of a promoter in front of *orf5* was also predicted by the above-described subcloning experiments.

We also performed Northern blot hybridization with the *orf5*-coding region as the ^{32}P probe. In addition to the 3.5-kb signal, a 2.2-kb signal was detected with the mRNA isolated from strain HH1 grown in the presence of A-factor (Fig. 3A). No signal was detected with the mRNA from strain HH1. The 3.5-kb signal supposedly corresponded to the mRNA initiating at P_{ORF5} and covering *orf5-orf4-amfR*. The 2.2-kb signal presumably represented the mRNA that started at P_{ORF5} and terminated somewhere at the 5′ portion of *amfR* (Fig. 1A).

The transcriptional organization of *orf5-orf4-amfR* was also confirmed by promoter-probing experiments with pTMA1 containing the malate dehydrogenase (*mdh*) gene as the reporter (data not shown). The *Eco*RI-*Eco*47III fragment containing the initiation codon of *orf5* expressed high MDH activity at 24 h (mid-exponential phase) and 48 h (early stationary phase) after inoculation, whereas the *Bam*HI-*Eco*47III fragment containing the initiation codon of *amfR* expressed only very low, but distinct, MDH activity. The MDH activity expressed by the latter was less than 1/10 of that of the former. The weak promoter activity in the *Bam*HI-*Eco*47III fragment was undetectable in the Northern blot experiment (Fig. 3A). All of these transcriptional analyses of the *orf5-orf4-amfR* region showed that *amfR* was transcribed mainly by P_{ORF5}.

S1 nuclease mapping. The above results indicated that the major promoter activity responsible for the transcription of *orf5-orf4-amfR* was located in front of *orf5* (P_{ORF5}). The transcriptional start site of P_{ORF5} was determined by S1 nuclease mapping. We used the 320-bp *Eco*RI-*Eco*47III fragment including 145 bp of the upstream region from the putative translational start site for ORF5 as the probe by end labeling it at the *Eco*47III cleavage terminus and hybridized it to mRNAs isolated from wild-type *S. griseus* IFO 13350 and strain HH1. A single transcriptional start site was detected with the mRNA isolated from the wild-type strain 24 h (mid-exponential phase) and 48 h (early stationary phase) after inoculation but not with that from strain HH1. For clearer resolution of the transcriptional start point, we also used the 130-bp probe (Fig. 3B). The start site was a G that was 72 bp upstream from the putative

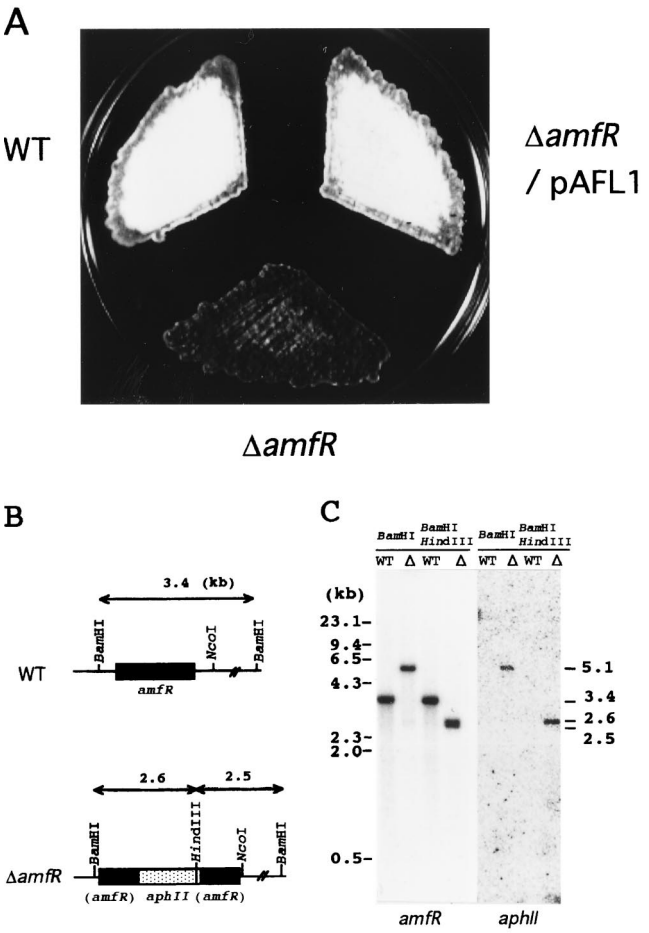

FIG. 2. Phenotypes of the *amfR* disruptant (A), schematic representation of the disrupted *amfR* (B), and Southern hybridization analysis of the chromosome of the *amfR* disruptant (C). (A) Patches were photographed after 5 days of growth at 28°C on YMPG medium. *S. griseus* IFO13350 (wild type [WT]) formed abundant spores, and the *amfR* mutant strain (Δ*amfR*) exhibited a Bld phenotype. The *amfR* disruptant harboring plasmid pAFL1 carrying the intact *amfR* gene with its upstream promoter region restored aerial mycelium and spore formation as abundantly as the wild-type strain. (B) Chromosomal *amfR* is disrupted by *aphII*. (C) The chromosomal DNAs from strain IFO 13350 (WT) and the *amfR* disruptant (Δ) were digested with *Bam*HI or *Bam*HI plus *Hin*dIII and hybridized with the *amfR* and *aphII* probes.

FIG. 1. Restriction map of the 6.5-kb region containing *orf5-orf4-amfR-amfA* and nucleotide sequences of part of this region. (A) The directions and extents of the ORFs deduced from the nucleotide sequence are shown just below the restriction map. The positions of a pair of the primers used for probes for Northern hybridization (Fig. 3) are indicated. The fragments on a low-copy-number plasmid and their ability to restore aerial mycelium formation in *S. griseus* HH1 are shown. (B) The nucleotide sequences of the region including the promoter in front of *orf5*, the termination codon of *orf5* and the initiation codon of *orf4*, and the termination codon of *orf4* and the initiation codon of *amfR*. Probable ribosome-binding sites for *orf5* and *amfR* are underlined. The transcriptional start point is indicated as +1. Numbers with an arrow indicate the probes used for the gel mobility shift assay (Fig. 4).

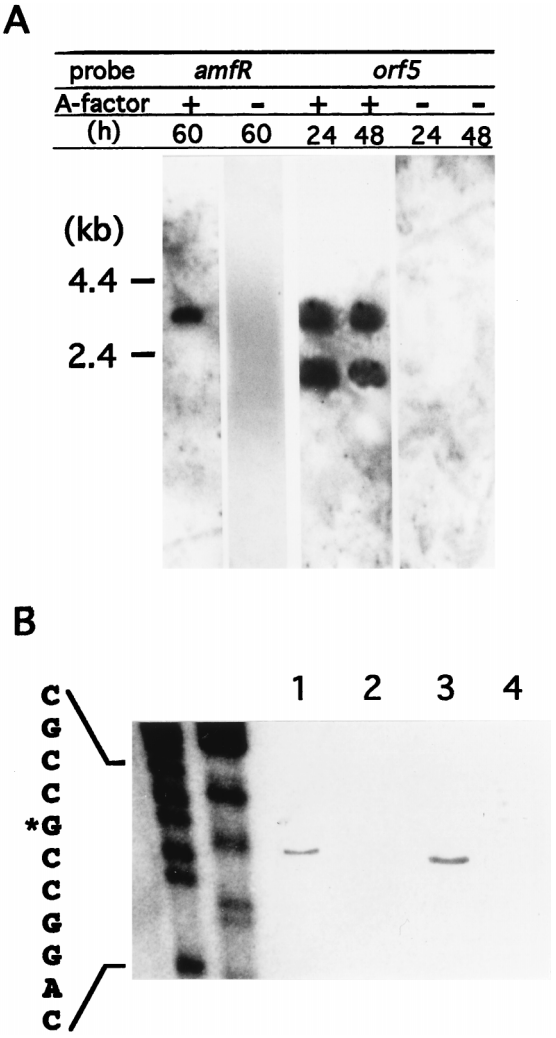

FIG. 3. Northern blot hybridization for detection of transcripts in the *orf5-orf4-amfR* region (A) and S1 nuclease mapping for determination of the transcriptional start point in the presence and absence of A-factor (B). *S. griseus* HH1 (A-factor negative) and the wild-type strain IFO 13350 (A-factor positive) were grown for the indicated periods. Total RNA was isolated from mycelium grown for the indicated period and subjected to Northern hybridization and S1 mapping. (A) When the *amfR* sequence (see Fig. 1) was used as the ^{32}P probe, a single transcript of 3.5 kb was seen in the mycelium from the wild-type strain but not from strain HH1. When the *orf5* sequence (see Fig. 1) was used, two transcripts of 3.5 and 2.2 kb were detected in the mycelium from the wild-type strain but not from strain HH1. (B) The amounts of transcripts directed by P_{ORF5} were examined by S1 nuclease mapping with mRNAs isolated from the wild-type and HH1 strains. The mRNAs were prepared from the wild-type strain grown for 24 h (lane 1) and 48 h (lane 3) and from strain HH1 grown for 24 h (lane 2) and 48 h (lane 4). Protected fragments were analyzed in parallel with sequencing ladders (lanes: left, T plus C; right, A plus G).

initiation codon. The presence of the transcript only in the wild-type strain, i.e., in the presence of A-factor, was in agreement with the results of the Northern blot analysis. The identical transcriptional start site was also detected by primer extension analysis with an 18-bp primer (nucleotide positions +106 to +89) (data not shown).

Detection of an A-factor-responsive DNA-binding protein. The Northern blot analysis as well as the S1 nuclease mapping experiments indicated that the major transcript covering *amfR* initiated at P_{ORF5} in positive response to A-factor. Because transcriptional control is often mediated by transcriptional factors that bind operator sites, we examined the presence of an A-factor-dependent DNA-binding protein(s) able to bind the P_{ORF5} sequence. Gel mobility shift assay with the 60-bp *Eco*RI-*Mlu*I fragment (Fig. 1) as the ^{32}P-labeled probe and with cell extracts of strain HH1 cultivated in the presence or absence of exogenously supplied A-factor as a protein source was used for detection of the putative DNA-binding protein. The cell extracts were fractionated by DEAE-Toyopearl column chromatography, and each fraction was subjected to the binding assay. A mobility shift was observed only with the cell extract of strain HH1 cultivated in the absence of exogenous A-factor (Fig. 4), although the gel shift patterns were disturbed due to proteins in various amounts in each fraction. In contrast, no significant shift bands were observed with the cell extract that was prepared from strain HH1 cultivated with exogenous A-factor. We also tested the cell extract similarly prepared from the wild-type strain of *S. griseus* and found that no significant retardation occurred (data not shown). As described below, the protein causing this mobility shift was found to bind specifically the P_{ORF5} sequence. These results showed that a DNA-binding protein that specifically bound P_{ORF5} is present in strain HH1, an A-factor-deficient mutant, and its production or DNA-binding activity is repressed by exogenously supplemented A-factor.

Purification of the A-factor-responsive DNA-binding protein. We purified the A-factor-responsive DNA-binding protein (tentatively named AdpB) by monitoring the ability to bind P_{ORF5}. Starting with mycelium ([wet] weight, 300 g) of strain HH1 grown at 30°C in 15 liters of YMPG medium, we purified AdpB (1.2 mg) to homogeneity by ammonium sulfate fractionation, three steps of column chromatography, and elution from nondenatured polyacrylamide gel. The affinity column chromatography on heparin was very useful, removing most proteins (Fig. 5, lane 5). The sample containing three major proteins eluted from the heparin column was subjected to native PAGE (lane 7) in order to identify AdpB by Southwestern blotting analysis with the same ^{32}P-labeled probe as in the gel mobility shift assays (lane 8). We eluted the protein giving a positive signal from a gel slice and ran it in SDS-PAGE (lane 6). AdpB purified to homogeneity in this way had an apparent molecular mass of 34 kDa.

Our attempt to determine the molecular mass of native AdpB by gel filtration failed, because AdpB appeared to form aggregates readily and eluted in the flowthrough fraction containing miscellaneous proteins with masses of more than 200 kDa. Almost no protein peaks were detected in other fractions examined by spectrophotometry. It is likely that the proteins eluted in the flow-through fraction represent aggregated forms of AdpB. Although the aggregated AdpB protein supposedly had much less activity, its DNA-binding activity, as determined by the gel mobility shift assay and the runoff transcription assay, was detectable as described below.

Gel mobility shift with the purified protein. Figure 6 shows the results of a gel shift assay for determination of the DNA-binding activity of the purified AdpB protein. The purified AdpB caused distinct retardation of the 158-bp *Eco*RI-*Sau*3AI fragment including the *Eco*RI-*Mlu*I region (lane 1). The binding specificity of AdpB was confirmed by addition of a 100-fold molar excess of cold probe DNA to the reaction mixture, which resulted in the abolishment of the shifted band (lane 5). We also used ^{32}P-labeled probes with various lengths, but none of these were retarded by AdpB (Fig. 5, lanes 2 to 4). The position of the 5′ end of each probe is indicated in Fig. 1B. These results indicate that the sequence essential for the binding of AdpB is located within the 28-bp region between −72 and −44 bp with respect to the transcriptional start point (Fig. 1B).

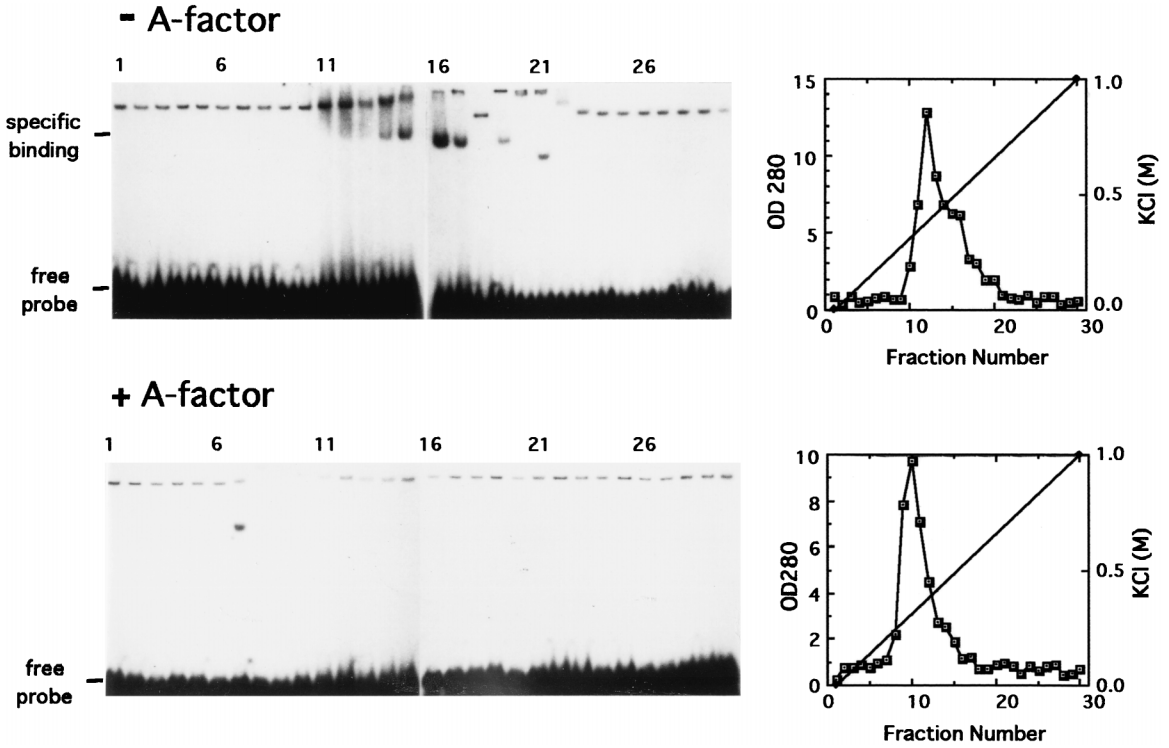

FIG. 4. Gel mobility shift assays for detection of a protein able to bind the promoter region of *orf5-orf4-amfR*. Crude extracts were prepared from the A-factor-deficient mutant strain *S. griseus* HH1 (upper panel) and strain HH1 grown in the presence of A-factor (lower panel) and fractionated by DEAE column chromatography. Each of the fractions was assayed by gel mobility shift assay with the 62-bp *Eco*RI-*Mlu*I fragment as the probe. OD 280, optical density at 280 nm.

Runoff transcriptional analysis of the A-factor-dependent promoter. The effect of AdpB on the transcription from P_{ORF5} was determined by runoff transcriptional assay. The RNA polymerase holoenzyme, mainly with σ^B promoter specificity partially purified from vegetative mycelium of *S. griseus* (28), was used. As for templates, we used two different DNA fragments containing the possible AdpB binding sequence and the transcriptional start site, as shown in Fig. 7A. The 316-bp *Eco*RI-*Eco*47III fragment (template *a*) and the 249-bp *Eco*RI-*Ava*II fragment (template *b*) were expected to produce transcripts of 244 and 177 nucleotides, respectively. With template *a*, a major transcript with the expected size was seen, and the amount of the transcript decreased as the amount of AdpB increased (Fig. 7B). With template *b*, a transcript with the expected size was seen in the absence of AdpB, and the presence of AdpB in the reaction mixture almost completely abolished it. These results clearly indicate that AdpB represses the in vitro transcription from P_{ORF5}, probably by binding to the operator sequence in P_{ORF5}.

DISCUSSION

The present study has demonstrated that *amfR* is essential for morphogenesis in *S. griseus* and is under the control of A-factor. Disruption of chromosomal *amfR* resulted in the complete abolishment of aerial mycelium formation, giving a Bld phenotype, which indicates its essential role in the onset of aerial mycelium formation. Nucleotide sequencing and transcriptional analysis of *amfR* showed that it was mainly transcribed by an A-factor-dependent promoter (P_{ORF5}) in front of the *orf5-orf4-amfR* operon. On the basis of the present study, we can hypothesize the regulation of *amfR* as follows. In the absence of A-factor, P_{ORF5} does not initiate transcription be-

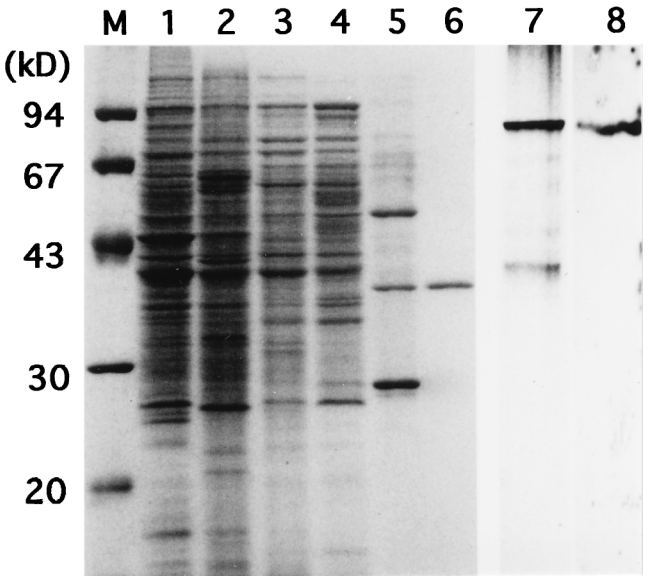

FIG. 5. SDS-PAGE used for monitoring purification of AdpB and Southwestern blotting with the active fraction after heparin affinity column chromatography. Lanes M to 6, SDS-PAGE; lanes 7 and 8, native PAGE. The molecular size markers in lane M were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa). Lane 1, the cell lysate from *S. griseus* HH1 (1 mg of protein); lane 2, after ammonium sulfate fractionation (1 mg of protein); lane 3, after DEAE-Toyopearl chromatography (0.5 mg of protein); lane 4, after Mono Q chromatography (0.5 mg of protein); lane 5, after heparin affinity chromatography (0.2 mg of protein); lane 6, after elution from nondenaturing polyacrylamide gel (0.1 mg of protein); lane 7, nondenaturing gel electrophoresis of the active fraction after heparin affinity chromatography; lane 8, autoradiogram of the same sample as in lane 7 subjected to Southwestern blotting with the ^{32}P-labeled *Eco*RI-*Eco*47III fragment.

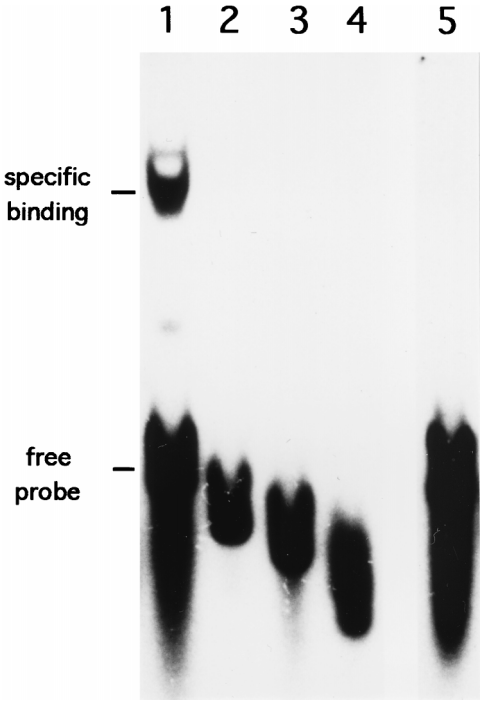

FIG. 6. Gel mobility shifts of the regions upstream of *orf5* caused by the purified AdpB protein. The 158-bp *Eco*RI-*Sau*3AI fragment and a series of fragments trimmed from the *Eco*RI end (see Fig. 1) were used. Lane 1, the 158-bp fragment (nucleotide positions −71 to 86 in Fig. 1B); lane 2, the 130-bp fragment (positions −43 to 86); lane 3, the 120-bp fragment (positions −34 to 86); lane 4, the 109-bp fragment (positions −22 to 86); lane 5, the 158-bp fragment in the presence of a 100-fold molar excess of the cold probe.

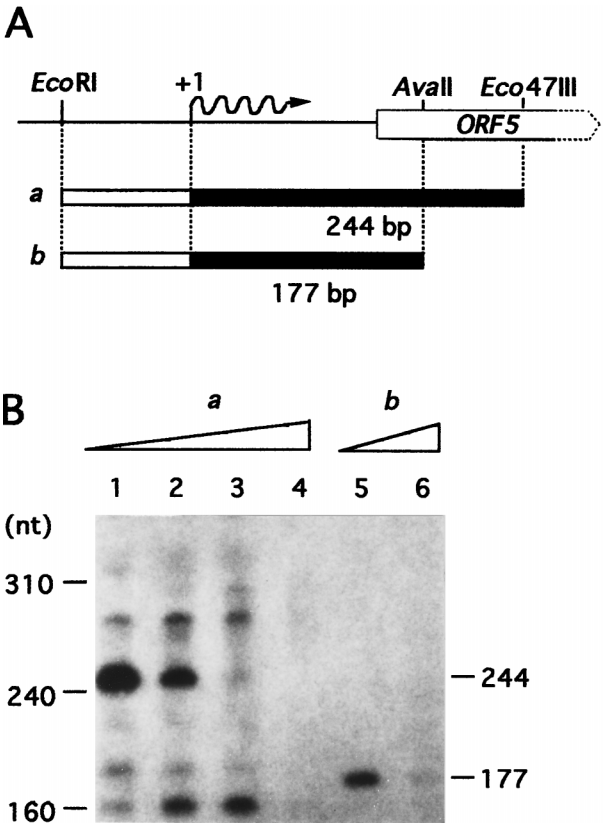

FIG. 7. In vitro transcription from P_{ORF5}. (A) Physical map of the DNA fragments used as the templates for runoff transcription. The *Eco*RI-*Eco*47III and the *Eco*RI-*Ava*II fragments were 316 and 249 bp, respectively. The expected transcripts, 244 and 177 nucleotides (nt) are depicted as solid bars. (B) The transcripts obtained with the two templates (*a* in lanes 1 to 4 and *b* in lanes 5 and 6) in the absence of AdpB (lanes 1 and 5) and the presence of 0.1 μg (lane 2), 1 μg (lane 3), or 10 μg (lanes 4 and 6) of AdpB were analyzed by 8 M urea-PAGE.

cause of the presence of AdpB that binds the operator site in P_{ORF5} and acts as a repressor. The AdpB binding site is contained in the region from −72 to −44 bp to the transcriptional start point, suggesting that AdpB prevents the RNA-polymerase holoenzyme from recognizing the −35 region and initiating transcription from this promoter. Since the AdpB binding site is an unusual location for an operator site, it could be part of a more complex region; for example, there might be an additional site(s) elsewhere in this region, so that repression could involve DNA looping. The presence of A-factor causes AdpB or its ability to bind P_{ORF5} to disappear, as determined by gel retardation assays, thus leading to transcription starting at P_{ORF5} and reading through into *amfR*. How the A-factor signal represses the biosynthesis of AdpB or inhibits the repressor-like function of AdpB is unclear. It is apparent that AmfR is a member involved in a decisive step in the A-factor regulatory cascade, leading to the onset of aerial mycelium formation. The details of the mechanism by which AdpB represses P_{ORF5} as a transcription factor will be elucidated when a large amount of AdpB is available by means of recombinant DNA techniques. The mechanism by which the A-factor signal causes DNA-bound AdpB to disappear will also be made clear by examination of the expression of *adpB*.

AdpB is distinct from ArpA, the A-factor receptor protein which serves as a repressor for both aerial mycelium formation and streptomycin biosynthesis (25). The apparent molecular masses of AdpB and ArpA are 34 and 29 kDa, respectively. The operator site of P_{ORF5} contains no ArpA recognition sequence (26); the consensus sequence recognized and bound by ArpA forms a palindrome 22 bp in length, and one-half of the palindrome is 5′-GG(T/C)CGGT(A/T)(T/C)G(T/G)-3′. In fact, AdpB did not bind the consensus 22-bp oligonucleotide in a gel mobility shift assay (data not shown).

Restoration of aerial mycelium formation by introduction of *amfR* together with P_{ORF5} on a low-copy-number plasmid into A-factor-deficient mutants can be explained in terms of a gene dosage effect; an increase in the copy number of *amfR* of even one or two leads to production of AmfR in a larger amount simply due to the gene dosage effect and to titration of AdpB. The failure of pSL6 containing *amfR* and its own promoter (P_{AmfR}) to cause aerial mycelium formation in strain HH1 may be due to a very weak promoter activity. Since *amfR* with P_{ORF5} cannot be placed on a high-copy-number plasmid, a moderate amount of AmfR or a phosphorylated form of AmfR appears to be important for healthy growth and normal morphogenesis. The phosphorylated form of AmfR is supposed to be active for the onset of morphogenesis, as was implied through site-directed mutagenesis of an aspartate residue to be phosphorylated (30).

We previously observed that *amfR-amfA* on the low-copy-number plasmid pTMA1 restored sporulation in strain HH1 (30). In the present study, pSL6 containing only *amfR* failed to suppress the aerial mycelium-defective phenotype, meaning that an extra copy of *amfA* compensates for the weak expression of *amfR*. Although the functions of *amfA* and *amfB*, both encoding a membrane translocator, are unclear, these genes may be somehow involved in aerial mycelium formation in

combination with *amfR*. Ma and Kendall (21) also observed that both a response regulator-like protein, RamR, and a membrane translocator-like protein, RamA or RamB, are essential for the acceleration of aerial mycelium formation in *S. coelicolor* A3 (2).

The operon structure of *orf5-orf4-amfR* suggests a functional relationship among the products of these genes, although the phenotypes conferred by pAFL1 and pAFL1Δ on strain HH1 are apparently the same. In addition, no changes in the phenotype of the wild-type strain harboring these plasmids are detectable. Nevertheless, it seems possible that ORF5 and ORF4 are involved in the subtle modulation of AmfR in some unknown way under certain growth conditions. Unlike typical two-component regulatory genes, which are closely encoded and cotranscribed, neither ORF5 nor ORF4 has homology with a series of kinases or phosphotransferases.

The fact that *amfR* encoding a protein belonging to the regulator family of the two-component regulatory systems plays a decisive role in the initiation of aerial mycelium formation is reminiscent of a similarity to *spo0A* required for the initiation of sporulation in *Bacillus subtilis* (10). Transcription of *spo0A* is negatively controlled by a repressor protein, AbrB, which determines the intracellular concentration of Spo0A. Spo0A is the final target of phosphorylation mediated by a so-called phosphorelay, and an increased ratio of the phosphorylated form of Spo0A is supposed to finally determine the onset of sporulation (27). As was implied by our mutational study of a putative phosphorylation site (30), phosphorylation of AmfR is supposed to be essential for its function as a transcriptional regulator. Like Spo0A in *B. subtilis* (10, 27), AmfR may play a role in widely receiving various signals transmitted through phosphoryl transfers and integrating them into the activation of the downstream-specific genes required for differentiation through its transcriptional control activity, finally leading to the onset of aerial mycelium formation. The precise mechanism can be elucidated by examination of the mode of phosphorylation of AmfR, which is probably mediated by several phosphotransferases encoded by different loci on the chromosome, as in *B. subtilis*.

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