JOURNAL OF BACTERIOLOGY, May 1993, p. 2652–2661
0021-9193/93/092652-10$02.00/0

Vol. 175, No. 9

Detection of an A-Factor-Responsive Protein That Binds to the Upstream Activation Sequence of *strR*, a Regulatory Gene for Streptomycin Biosynthesis in *Streptomyces griseus*

DUSICA VUJAKLIJA, SUEHARU HORINOUCHI,* AND TERUHIKO BEPPU

Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Received 26 June 1992/Accepted 26 February 1993

DNA-binding assays using mobility shift polyacrylamide gel electrophoresis revealed the presence of a protein that specifically bound to a restriction fragment −288 to −191 bp upstream from the transcriptional start point of *strR*, a regulatory gene for streptomycin biosynthesis in *Streptomyces griseus*. The binding site corresponded to an upstream activation sequence predicted from the results of in vivo promoter assays. The binding was greatly enhanced by 5 mM Mg^{2+}. This binding was detected with the protein source only from the wild-type strain and not from an A-factor-deficient mutant strain. The exogenous supplementation of A-factor to the A-factor-deficient mutant strain caused the appearance of the protein in the DNA-binding assay. A synthetic nucleotide 52 bp in length (region from −293 to −242), which was synthesized on the basis of data obtained from both retardation assays with dissected DNA fragments and in vivo promoter assays, was retarded by the A-factor-dependent protein. In addition to this A-factor-dependent protein, at least three proteins with different recognition site affinities capable of binding to the upstream region of the *strR* promoter were detected. The binding of one of these proteins to both sides of the upstream activation sequence bound by the A-factor-dependent protein was completely abolished in the presence of ATP and Mg^{2+} in the incubation mixture. The region bound by these proteins showed anomalous electrophoretic mobility, like that of a bent DNA molecule, which is probably caused by the presence of many blocks consisting of A and T. The region bound by these proteins was found to be transcribed in the orientation opposite to that of *strR*.

A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) (17) is a microbial hormone controlling streptomycin production, resistance to streptomycin, and aerial mycelium formation in *Streptomyces griseus* (5, 9). Although A-factor itself seems to exert its regulatory function only in *S. griseus*, there is a possibility that γ-butyrolactones structurally similar to A-factor serve as hormonal regulators in general in a wide variety of *Streptomyces* spp. (6). Our discovery of the A-factor receptor protein, which appears to act as a repressor-type regulator, has led to the idea that A-factor binds to the receptor protein as an early event in the A-factor regulatory cascade, resulting in derepression of a still unknown key gene(s) that is important for secondary metabolism and cell differentiation in *S. griseus* (14, 15). Streptomycin biosynthesis is one of the phenotypes that is expressed through the A-factor regulatory cascade. We previously identified an A-factor-dependent promoter in the streptomycin biosynthetic gene cluster of *S. griseus* (25). The promoter responsible for cotranscription of *strR* encoding a regulatory protein and *aphD* encoding the major streptomycin resistance determinant, i.e., streptomycin-6-phosphotransferase, was activated only in the presence of A-factor. The region essential for its A-factor dependence was determined to be located upstream from the transcriptional start point of *strR*. In addition, experiments with a high-copy-number plasmid predicted the presence of a putative activator protein which might bind to this region (25).

We therefore conducted experiments to detect the predicted activator protein that might bind to the region upstream from the *strR* promoter. Partial fractionation of the crude extract of *S. griseus* allowed us to detect a protein able to bind to the predicted region by a DNA-binding assay using mobility shift polyacrylamide gel electrophoresis. The binding by this protein was observed with the extract only from the wild-type strain and not from an A-factor-deficient mutant strain. In this article, we describe the A-factor-responsive protein capable of binding to the region upstream from the *strR* promoter, which we believe is a useful step for understanding the regulation of streptomycin biosynthesis through the A-factor regulatory cascade. During the course of this study, we also found that, in addition to the A-factor-responsive binding protein, many different proteins were capable of specifically binding to the upstream region of *strR*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used are listed in Table 1. *S. griseus* strains were grown in YMPG medium (pH 7.2) containing the following, in grams per liter: yeast extract (Difco Laboratories), 2; meat extract (Wako Pure Chemicals), 2; Bacto Peptone (Difco), 4; NaCl, 5; $MgSO_4 \cdot 7H_2O$, 2; glucose, 10. Growth conditions for *Escherichia coli* strains were as described by Maniatis et al. (12).

Enzymes and materials. Restriction endonucleases, DNA polymerase I (Klenow fragment), T4 DNA ligase, T4 polynucleotide kinase, and bacterial alkaline phosphatase were purchased from Takara Shuzo Co. or Boehringer-Mannheim and were used according to the recommendations of the suppliers. [α-^{32}P]dATP (3,000 and 6,000 Ci/mmol) and [γ-^{32}P]ATP (6,000 Ci/mmol) were obtained from Amersham Corp. ATP, CTP, GTP, UTP, cyclic AMP, and poly(dI-dC) · poly(dI-dC) were purchased from Pharmacia, Inc. DEAE membrane NA45 was from Schleicher & Schuell

* Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
S. griseus IFO 13350	Wild-type	IFO[a]
S. griseus HH1	A-factor-deficient mutant strain derived from IFO 13350 by incubation at 37°C; non-streptomycin producing, sporulation negative	8
E. coli JM109	*recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi-1 hsdR17 relA1 supE44* F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*	Takara Shuzo Co.
E. coli TG1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*	12
Plasmids		
pUC19	Carries ampicillin resistance	26
pUC19-A	Contains the 637-bp *Nru*I fragment (Fig. 1) and part (350 bp) of the *mdh* gene	25
pUC19-206	Contains the 206-bp *Taq*I fragment (Fig. 1) at the *Acc*I site in the polylinker of pUC19	This study
pUC19-214	Contains the 214-bp *Taq*I fragment at the *Acc*I site of pUC19	This study

[a] Institute of Fermentation, Osaka, Japan.

Corp. A Geneclean kit was from Bio 101, Inc. Centricut 10 was from Kurabo, Tokyo, Japan.

Recombinant DNA studies. General techniques for recombinant DNA work were described by Maniatis et al. (12). DNA fragments in agarose gel slices were recovered by electrophoresis onto DEAE-cellulose papers (4) or with a Geneclean kit according to the recommendation of the supplier. DNA fragments in polyacrylamide gel slices were eluted in high-salt buffer (1.0 M NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA) by the method of Maniatis et al. (12). Nucleotide sequences were determined by the M13-dideoxynucleotide method with M13mp18 and M13mp19 (20).

Partial purification of DNA-binding proteins. A 3-day-old culture of *S. griseus* IFO 13350 or an A-factor-deficient mutant strain, *S. griseus* HH1, was diluted 1:100 into fresh YMPG medium and grown aerobically at 30°C for 60 h on a reciprocal shaker. When necessary, chemically synthesized A-factor (18) was added to the culture at a final concentration of 100 μg/ml. This and all subsequent steps were performed at 4°C. Protein concentrations were measured by spectrophotometry.

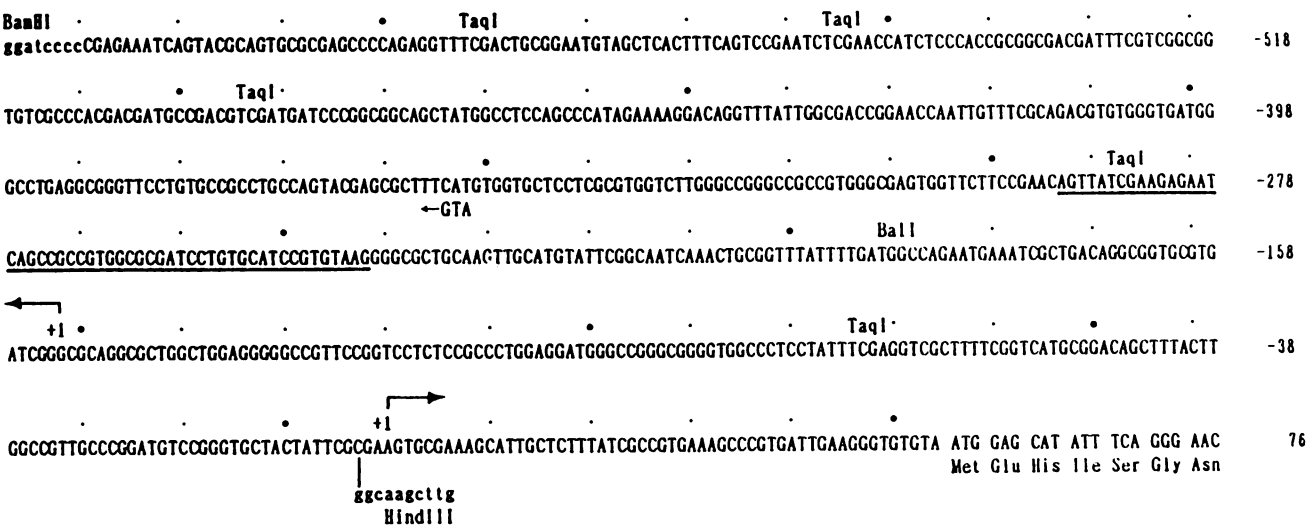
(i) Preparation of crude extracts. Mycelium (wet weight, 20 to 25 g) harvested by centrifugation from 500 ml of culture was washed once with standard buffer (10 mM Tris-HCl [pH 7.0], 1 mM EDTA, 1 mM dithiothreitol, 10% [vol/vol] glycerol) and suspended in 100 ml of the same buffer. The mycelium was disrupted with a French pressure cell by two passages at about 1,000 lb/in^2. Cell debris was removed by centrifugation at 55,000 × *g* for 1 h. Proteins in the supernatant fluid that had been precipitated with ammonium sulfate at 60% saturation were collected by centrifugation at 55,000 × *g* for 30 min. The precipitate was dissolved in 50 ml of standard buffer and dialyzed against 6 liters of the same buffer overnight, with two changes of buffer.

(ii) DEAE-Toyo Pearl column chromatography. Twenty-five milliliters of the dialyzed sample was applied to a DEAE-Toyo Pearl column (3 by 7 cm; Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan) previously equilibrated with standard buffer. After the column had been washed with 200 ml of the same buffer, proteins were eluted step-wise with 200 ml each of the buffer with five different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 M) of KCl. Peak fractions (about 150 ml) from each step were pooled, concentrated with a Centricut apparatus, and examined for DNA-binding activities.

Restriction fragments and an oligonucleotide for DNA-binding assays. The DNA fragments cloned on pUC19-A, pUC19-206, and pUC19-214 were used as the starting materials for the preparation of ^{32}P-labeled fragments. Restriction fragments were ^{32}P labeled by phosphorylating the 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase, separated by 10% polyacrylamide gel electrophoresis, and eluted in high-salt buffer. A 52-mer ^{32}P-labeled oligonucleotide was prepared by annealing two single-stranded DNA of 52 and 46 nucleotides (nt) (5′-AGTTATCGAAGAGAATCAGCCGC CGTGGCGCGATCCTGTGCATCCGTGTAAG-3′ and 5′-CTTACACGGATGCACAGGATCGCGCCACGGCGGCT GATTCTCTTCG-3′) synthesized on a Beckman System Plus 1E plus DNA synthesizer, ^{32}P labeled by filling in recessed ends with [α-^{32}P]dATP and Klenow fragment, and then recovered by polyacrylamide gel electrophoresis.

Electrophoretic mobility shift assays. DNA-binding assays by mobility shift assays were done essentially by the method of Chodosh (1). 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 20 min in a buffer containing 10 mM Tris-HCl (pH 7.8), 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 30 μl. When necessary, cation-yielding compounds ($MgCl_2$, $CaCl_2$, and $ZnCl_2$) and nucleotides (ATP, GTP, CTP, UTP, and cyclic AMP) were added to the reaction mixture. After incubation, complexes and free DNA were resolved on nondenaturing polyacrylamide gels containing 6% acrylamide and 0.075% bisacrylamide with a running buffer containing 40 mM Tris-HCl (pH 7.8), 20 mM sodium acetate, and 1 mM EDTA. Gels were dried and subjected to autoradiography with a Du Pont Cronex intensifying screen.

Primer extension. *S. griseus* IFO 13350 was grown at 28°C for 3 days in 10 ml of YMPG medium. After disruption of mycelial clumps with a glass homogenizer, 1 ml was inoculated into 100 ml of fresh YMPG medium and grown at 28°C for 2 days. The mycelium was harvested by centrifugation. Total cellular RNA was prepared as previously described (25). A 40-mer oligonucleotide, 5′-GCAGACGTGTGGGT GATGGGCCTGAGGCGGGTTCCTGTGC-3′ complementary to a sequence in the putative coding region of the *strD* gene (nt −416 to −377 with respect to the transcriptional start point of *strR*; see Fig. 1), was synthesized on an Applied Biosystems 380A DNA synthesizer and labeled at the 5′ end with T4 polynucleotide kinase and



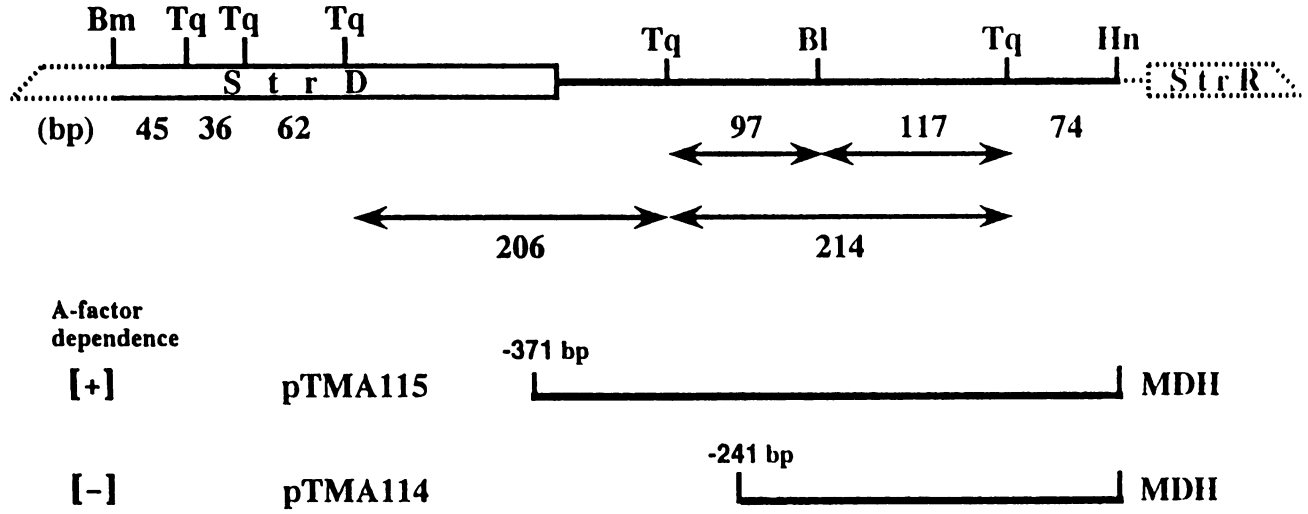


FIG. 1. Nucleotide sequence of the region upstream from the *strR* gene (top) and the restriction fragments used as probes for gel retardation assays (bottom). (Top) For experimental convenience, the original *Nru*I sites were changed into *Bam*HI and *Hin*dIII sites by attaching respective linkers, as shown. The transcriptional start point of *strR* (2, 25) is indicated by an arrow at nt +1. The Met at nt 55 to 57 is the translational start codon for *strR*. The arrow at nt −152 indicates a transcriptional start point for the *strD* gene encoding a putative hexose nucleotidylating enzyme, which was determined by primer extension as shown in Fig. 8. A presumptive initiation codon for *strD* is at nt −352 to −354. The underlined nucleotide sequence was used as the probe for detecting the A-factor-responsive binding protein (Fig. 3). (Bottom) The restriction fragments shown in base pairs and indicated by arrows were used as ^{32}P-labeled probes for gel retardation assays. Abbreviations: Bm, *Bam*HI; Tq, *Taq*I; Bl, *Bal*I; Hn, *Hin*dIII. Below the restriction map are shown the fragments cloned into promoter-probe plasmid pTMA1 containing a malate dehydrogenase (MDH) gene as the reporter gene (25). Transcription by the 372-bp fragment (nt −371 to +1) on pTMA115 was A-factor dependent, whereas that by the 242-bp fragment (nt −241 to +1) on pTMA114 was not.

[γ-^{32}P]ATP. Fifty or 100 μg of total cellular RNA and 20 ng of 5′-end-labeled oligonucleotide primer were heated at 80°C for 10 min and then at 42°C for 8 h in 30 μl of hybridization buffer [40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4), 1 mM EDTA, 0.4 M NaCl, 80% formamide]. Reverse transcription with 20 U of avian myeloblastosis virus reverse transcriptase was carried out at 42°C for 1 h in 20 μl of 50 mM Tris-HCl (pH 8.2)–50 mM KCl–10 mM $MgCl_2$–10 mM dithiothreitol–5 mM each deoxyribonucleotides–50 μg of actinomycin D per ml, essentially by the method of Maniatis et al. (12). The extended product was analyzed by electrophoresis on a 6% denaturing polyacrylamide gel. Dideoxynucleotide sequencing reaction mixtures primed from the oligonucleotide were electrophoresed in neighboring lanes.

RESULTS

Dissection of the upstream sequence of *strR* and protein fractionation. The nucleotide sequence of the region up-

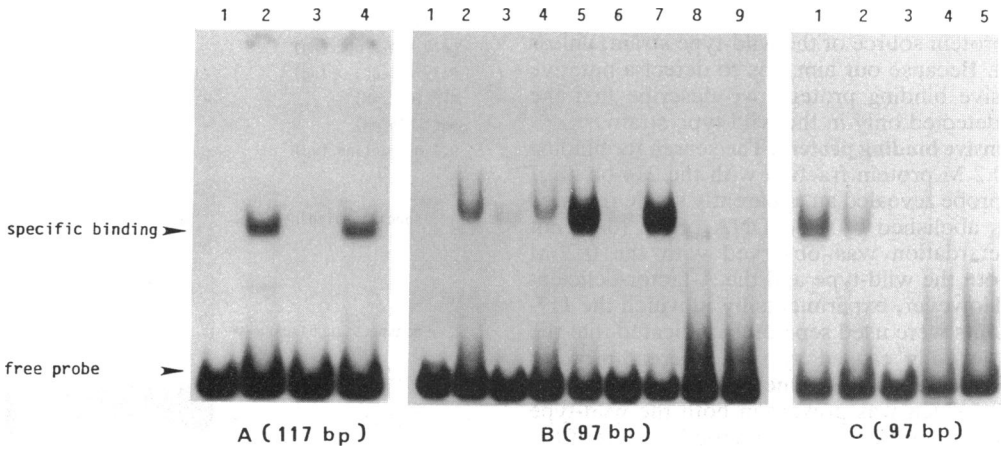


FIG. 2. Gel retardation analysis with a dissected 214-bp fragment and the 0.2 M protein fraction. (A) The combination of the 117-bp *Taq*I-*Bal*I fragment and the 0.2 M protein fraction from the wild-type strain yielded a DNA-protein complex. Lanes: 1, migration of the protein-free probe; 2, migration after incubation with the protein fraction in standard buffer; 3, the same as lane 2, except that a 20-fold molar excess of cold DNA probe was added; 4, the same as lane 2, except that 2 μg of poly(dI-dC) polymer was added. (B) The combination of the 97-bp *Bal*I-*Taq*I fragment and the 0.2 M fraction yielded a DNA-protein complex whose binding was enhanced by $MgCl_2$. Lanes: 1, migration of the free probe; 2, migration after incubation with the 0.2 M fraction from the wild-type strain in standard buffer; 3, the same as lane 2, except that a 20-fold molar excess of cold DNA probe was added; 4, the same as lane 2, except that 2 μg of poly(dI-dC) polymer was added; 5, the same as lane 2, except that $MgCl_2$ was added at a final concentration of 5 mM; 6, the same as lane 5, except that a 20-fold molar excess of cold DNA probe was added; 7, the same as lane 5, except that 2 μg of poly(dI-dC) polymer was added; 8, migration after incubation with the 0.2 M fraction from the A-factor-deficient mutant strain in standard buffer; 9, the same as lane 8, except that $MgCl_2$ was added at a final concentration of 5 mM. (C) The combination of the 97-bp *Bal*I-*Taq*I fragment and the 0.2 M fraction from the A-factor-deficient mutant strain grown in the presence of 100 μg of exogenous A-factor per ml yielded a DNA-protein complex. Lanes: 1, migration after incubation with the protein in a buffer containing 5 mM $MgCl_2$; 2, migration after incubation with the protein fraction in standard buffer; 3, free probe. Lanes 4 and 5 are control lanes: 4, migration after incubation of the 0.2 M fraction from the A-factor-deficient mutant strain grown without A-factor supplement in a buffer containing 5 mM $MgCl_2$; 5, the same as lane 4, except that incubation was done in standard buffer.

stream from the transcriptional start point of the *strR* promoter (25) cloned from *S. griseus* IFO 13350 was determined by the dideoxynucleotide method (Fig. 1). The sequence turned out to be almost identical to that of *S. griseus* N2-3-11 (2), except for the −476 and −475 positions. This sequence

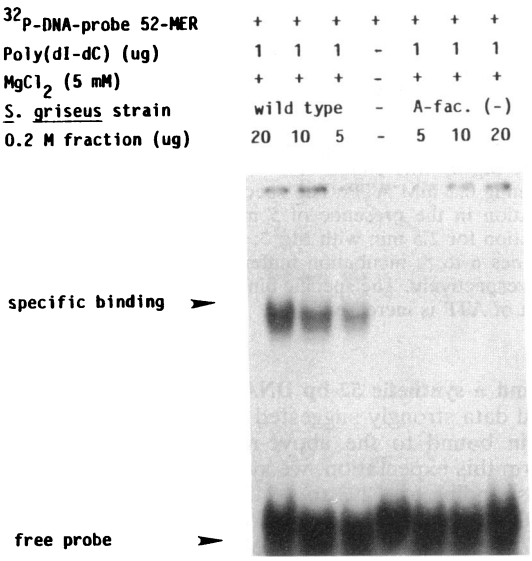


FIG. 3. Binding of the A-factor-dependent protein to a synthetic nucleotide 52 bp in length. The A-factor-dependent protein able to bind to the 52-mer nucleotide (−293 to −242 in Fig. 1) is present in the 0.2 M protein fraction prepared from the wild-type strain but not from the A-factor-deficient mutant strain.

contains an *Alu*I cleavage site, and we checked for the presence of an *Alu*I site by restriction analysis. The DNA fragment derived from this upstream region was used as a probe in the DNA-binding assays (Fig. 1). We first examined for the presence of proteins that caused a mobility shift on polyacrylamide gel electrophoresis with a crude extract of the wild-type *S. griseus* strain and the 637-bp *Bam*HI-*Hin*dIII fragment excised from pUC19-A. The experiments revealed the presence of multiple proteins able to bind specifically and nonspecifically to the probe (data not shown). It was difficult to analyze the complex banding pattern because of the appearance of multiple retarded bands. We therefore fractionated the crude extract as a protein source by DEAE-Toyo Pearl column chromatography. Two fractions that had been eluted at between 0.1 and 0.2 M KCl (referred to as the 0.2 M protein fraction) and between 0.2 and 0.3 M KCl (referred to as the 0.3 M protein fraction) were found to contain proteins able to bind specifically to the 637-bp probe. Other fractions showed no significant retardation. In the following experiments, the 0.2 M and 0.3 M protein fractions prepared from the A-factor-deficient mutant strain, *S. griseus* HH1, were similarly examined in parallel with those from the wild-type strain in order to detect an A-factor-responsive binding protein.

We also dissected the 637-bp DNA fragment into smaller parts to avoid the complexity in banding pattern. Before describing the details, we mention here that no retardation occurred in any combination with the 45-, 36-, and 62-bp DNA fragments as the probes and the two fractions from the wild-type and the A-factor-deficient mutant strains as the protein sources. All the following binding proteins, except for one protein able to bind to the 97-bp *Taq*I-*Bal*I fragment, were present in both the wild-type and the A-factor-deficient

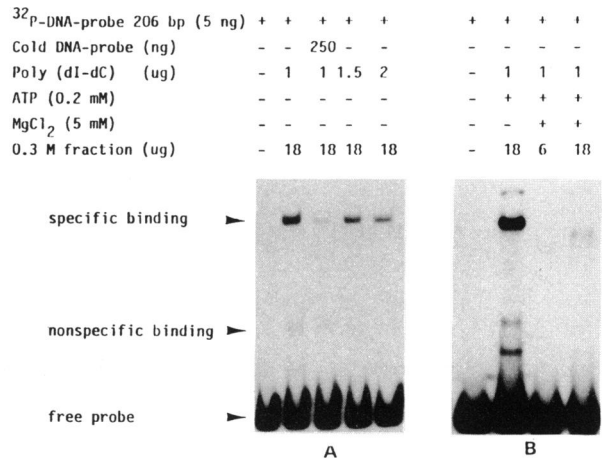
mutant strains. We therefore show retardation profiles obtained with the protein source of the wild-type strain, unless otherwise stated. Because our aim was to detect a putative A-factor-responsive binding protein, we describe first the binding protein detected only in the wild-type strain.

A-factor-responsive binding protein. The search for binding proteins in the 0.2 M protein fraction with the 214-bp *Taq*I fragment as the probe revealed an apparently single retarded band which was abolished by cold DNA probe (data not shown). This retardation was observed with the 0.2 M fractions from both the wild-type and the A-factor-deficient mutant strains. However, experiments by in which the 117- and 97-bp fragments were used separately indicated that the retardation was actually caused by two different proteins recognizing different sequences. One protein (shown by a circle in Fig. 10), which was present in both the wild-type and A-factor-deficient strains, caused a mobility shift of the 117-bp fragment (Fig. 2A, lane 2). This retardation was abolished by a cold DNA probe (lane 3) but not by a poly(dI-dC) polymer (lane 4). Cations (Mg^{2+}, Ca^{2+}, and Zn^{2+}) and nucleotides (ATP, GTP, UTP, and cyclic AMP) exerted no effect on this binding.

The other protein (shown by a checked wedge in Fig. 10) which bound to the 97-bp fragment was present only in the wild-type strain (Fig. 2B, lane 2) and not in the A-factor-deficient mutant strain, HH1 (lanes 8 and 9). This binding was enhanced by 5 mM $MgCl_2$ and was specific because a 20-fold molar excess of cold DNA abolished the binding. A faint band, seen in lanes 8 and 9, with a slightly faster mobility than the major retarded band is ascribable to a nonspecific binding protein whose binding is observed only in the absence of the protein responsible for the major retarded band. We next examined whether A-factor supplementation to the A-factor-deficient mutant strain restored the production of this protein. As expected, the 0.2 M protein fraction prepared similarly from strain HH1 grown in the presence of 100 μg of A-factor per ml caused a mobility shift (Fig. 2C). This binding was enhanced by 5 mM $MgCl_2$. A-factor supplementation to the incubation mixture containing the probe and the 0.2 M fraction from strain HH1 did not cause any mobility shift. All these data showed that the mobility shift was A-factor dependent, probably because the binding protein responsible for this shift was produced in *S. griseus* cells grown only in the presence of A-factor.

We previously reported that a putative A-factor-dependent activator protein bound to the region −330 to −430 bp upstream from the transcriptional start point of the *strR* promoter, on the basis of experiments to detect the promoter activity of truncated upstream regions of *strR* in the presence and absence of A-factor (25). We observed that the fragment on pTMA115 supposedly covering a part of the 206-bp fragment still showed A-factor-dependent transcription but that a shorter fragment on pTMA114 did not (Fig. 1). The 97-bp fragment, which was identified in the present work as the target of the A-factor-dependent protein, does not overlap with this region. To settle the discrepancy, we determined the exact ends of the 5′-flanking regions of the fragments on these plasmids by nucleotide sequencing. The 5′ ends of the fragments on pTMA114 and pTMA115 were determined to be −241 and −371, respectively. These results correct our previous assumption regarding the location of the position and show that the presumptive upstream activation sequence (UAS) bound by the putative activator protein is −371 to −241. This region shares 47 bp, from −288 to −242, with the 97-bp fragment identified here.

Gel retardation with the A-factor-responsive binding protein and a synthetic 52-bp DNA fragment. The above-mentioned data strongly suggested that the A-factor-responsive protein bound to the above-mentioned 47-bp region. To confirm this expectation, we synthesized a 52-bp nucleotide corresponding to −293 to −242 and used it as the probe in the DNA-binding assay. As shown in Fig. 3, a mobility shift was observed with the 0.2 M protein fraction from the wild-type strain but not from the A-factor-deficient mutant strain. This binding was abolished by an excess amount of unlabeled probe (data not shown). The extent of retardation increased as the amount of the protein source increased. A similar mobility shift was observed with the 0.2 M fraction



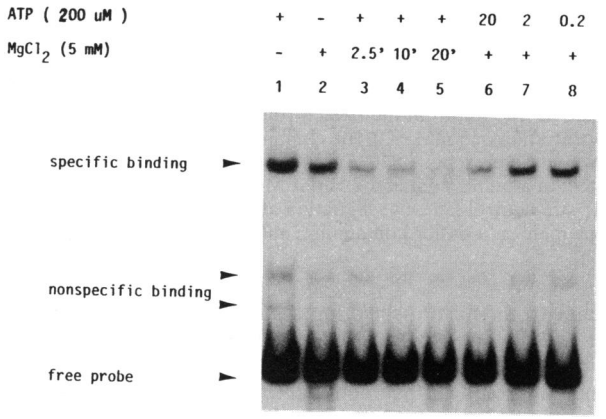


FIG. 4. Band shift analysis with the 206-bp *Taq*I fragment and the 0.3 M fraction (A) and characterization of its binding (B and C). (A) The retardation is abolished by a 50-fold molar excess of cold DNA but not by an excess amount of poly(dI-dC) polymer. (B) The retardation is not affected by ATP but is completely abolished by ATP plus Mg^{2+}. (C) The retardation is not affected by ATP (lane 1) or Mg^{2+} (lane 2). A slight decrease of the specific binding in lane 2 is perhaps due to DNases present in the protein fraction. For lanes 3 to 5, $MgCl_2$ was added at intervals to the incubation mixture containing 0.2 mM ATP. The specific binding is decreased as the incubation in the presence of 5 mM Mg^{2+} is prolonged (lane 3, incubation for 2.5 min with Mg^{2+}; lane 4, 10 min; lane 5, 20 min). For lanes 6 to 8, incubation buffers contained 20, 2, and 0.2 μM ATP, respectively. The specific binding is decreased as the concentration of ATP is increased.

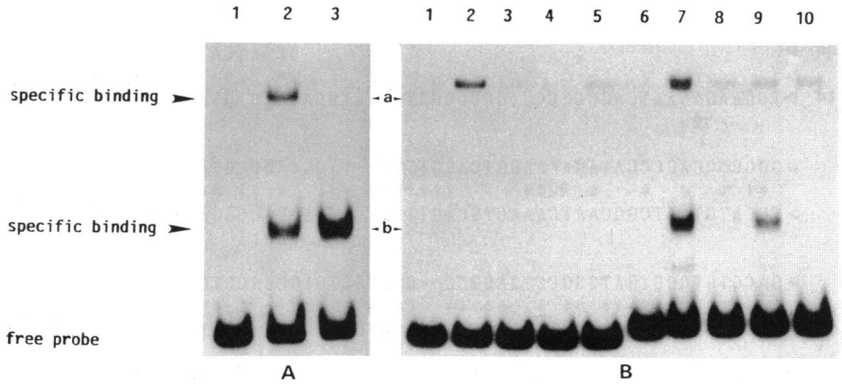


FIG. 5. Gel retardation analysis with the 214-bp fragment and the 0.3 M protein fraction (A) and characterization of the binding affinity (B). (A) The 0.3 M fraction prepared from the wild-type strain was assayed with the 214-bp *Taq*I fragment as the probe. Lanes: 1, control lane without the protein fraction; 2, two specific DNA-protein complexes (*a* and *b*) formed in standard buffer containing 0.1 mM ATP (a similar pattern was observed with the buffer without ATP); 3, a DNA-protein complex formed in standard buffer containing 0.1 mM ATP and 5 mM $MgCl_2$. In the presence of ATP and $MgCl_2$, band *a* disappeared whereas formation of the DNA-protein complex (band *b*) was greatly enhanced. (B) Binding affinities of the DNA-protein complexes. The 206-bp *Taq*I fragment (shown in lane 1) was mixed with the 0.3 M fraction in standard buffer containing 0.1 mM ATP (lane 2; the same as the second lane in Fig. 2B), 0.1 mM ATP and a 100-fold molar excess of the unlabeled 206-bp fragment (lane 3), 0.1 mM ATP and a 100-fold molar excess of the unlabeled 214-bp *Taq*I fragment (lane 4), or a 100-fold molar excess of the unlabeled 206-bp *Taq*I fragment without ATP (lane 5). The binding is abolished by both the 206- and 214-bp fragments. In the absence of ATP (lane 5), the extent of abolishment is slightly reduced. Reciprocal competition is shown from lanes 6 to 10. The 214-bp *Taq*I fragment (lane 6) was mixed with the 0.3 M fraction in a buffer containing 0.1 mM ATP (lane 7; same as lane 2 in panel A), 0.1 mM ATP and a 100-fold molar excess of the unlabeled 214-bp fragment (lane 8), 0.1 mM ATP and a 100-fold molar excess of the unlabeled 206-bp fragment (lane 9), or a 100-fold molar excess of the unlabeled 214-bp fragment without ATP (lane 10). The formation of band *b* is not abolished by the 206-bp fragment.

from strain HH1 grown in the presence of A-factor (data not shown). These data clearly indicated that the A-factor-responsive protein bound to the 52-bp fragment.

Additional proteins able to bind to the upstream region of the *strR* promoter. During our search for the above-mentioned A-factor-responsive protein, we found other species of binding proteins with different recognition site affinities. One was the above-mentioned protein present in the 0.2 M protein fraction which bound to the 117-bp *Bal*I-*Taq*I fragment. Two other proteins were found in the 0.3 M fraction from both the wild-type and the A-factor-deficient strains, as follows.

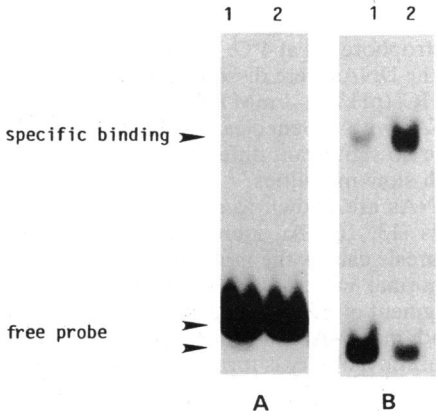


FIG. 6. Gel retardation analysis with parts of the dissected 214-bp fragment as probes. The ^{32}P-labeled probes used were obtained by cleavage of the 214-bp *Taq*I fragment with *Bal*I. (A) The 117-bp *Taq*I-*Bal*I fragment (lane 1) and the 0.3 M protein fraction yielded no retarded band (lane 2). (B) The 97-bp *Bal*I-*Taq*I fragment and the 0.3 M fraction yielded a DNA-protein complex (lane 1), and the binding was enhanced by 5 mM $MgCl_2$.

(i) ATP-Mg^{2+}-dependent binding protein. The experiments with the 206-bp *Taq*I fragment and the 0.3 M protein fraction from the wild-type strain revealed the presence of a protein (shown by a hexagon in Fig. 10) that caused a mobility shift in the DNA-binding assay (Fig. 4A). No retardation occurred with the 0.2 M protein fraction. A 50-fold molar excess of cold DNA as the competitor greatly reduced the binding, indicating that this binding was specific. This binding was not significantly affected by increased amounts of poly(dI-dC) · poly(dI-dC). The same retardation pattern was also observed with the 0.3 M fraction from the A-factor-deficient strain.

We examined the effects of 5 mM each cation-yielding compound ($MgCl_2$, $CaCl_2$, and $ZnCl_2$) and 0.2 mM each nucleotide (ATP, GTP, UTP, and cyclic AMP) on binding. These compounds did not affect binding when added separately (data not shown). Only in the presence of $MgCl_2$ did slight degradation of the DNA probe, probably due to DNases still present in the protein fraction, occur. However, the mobility shift was completely abolished in the presence of 200 μM ATP and 5 mM $MgCl_2$ (Fig. 4B). Neither the combination of ATP and other cation-yielding compounds nor the combination of $MgCl_2$ and other nucleotides affected the binding, although the nonspecific binding was slightly affected in a nonspecific way by these treatments (panels B and C). Because of this unexpected observation, we further examined the effect on the binding caused by the combination of ATP and $MgCl_2$ (Fig. 4C). As described above, $MgCl_2$ at 5 mM or ATP at 200 μM did not cause a significant effect. The slight decrease in the retarded signal in lane 2 was supposedly caused by DNases in the incubation mixture. When $MgCl_2$ at a final concentration of 5 mM was added at intervals to the incubation mixture containing 200 μM ATP, the binding was significantly repressed, even by the 2.5-min incubation in the presence of $MgCl_2$ (lane 3), just like the case in which $MgCl_2$ and ATP were added from the begin-

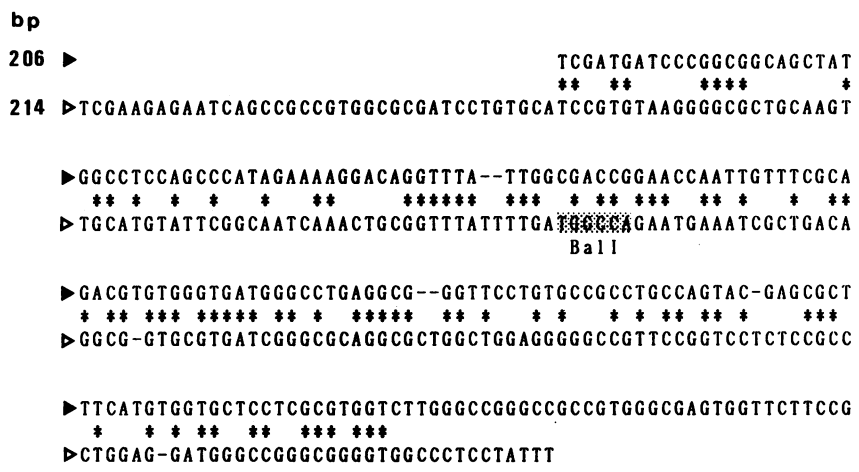


FIG. 7. Alignment of the nucleotide sequences of the 206- and 214-bp fragments. Identical nucleotides are marked by asterisks.

ning (Fig. 4B, lanes 2 and 3). The extent of inhibition was dependent on the concentration of ATP (lanes 5 to 8). The intensity of the retarded signal obtained from the mixture containing 20 μM ATP and 5 mM $MgCl_2$ (lane 6) was about 28% of that in the control assay (lane 1) when calculated by densitometry.

Concerning the binding site of the ATP-Mg^{2+}-dependent protein, we found that the protein bound to an additional site closer to the transcriptional start site of *strR*. Examinations with the 214-bp fragment and the 0.3 M protein fraction revealed the presence of two retarded bands, as shown in Fig. 5A. Both bands disappeared when a 100-fold molar excess of cold DNA was added as the competitor (data not shown). Similar retardation patterns were observed with the 0.3 M fraction from the A-factor-deficient mutant strain. Examinations of the effects of cations and nucleotides showed that band *a* behaved in the exactly same manner as the above-described retarded band dependent on ATP-Mg^{2+}. We then performed reciprocal competition experiments (Fig. 5B). Band *a*, which was retarded with the 214-bp fragment and the 0.3 M fraction (lane 7), was abolished by a 100-fold molar excess of the 206-bp fragment (lane 9), and the retardation caused by the 206-bp fragment and the 0.3 M fraction (lane 2) was abolished by a 100-fold molar excess of the 214-bp fragment (lane 4). On the other hand, the retardation of band *b* caused by the 214-bp fragment and the 0.3 M fraction was not significantly abolished by the 206-bp fragment (lane 9). We therefore concluded that the same protein in the 0.3 M fraction was capable of binding to two different positions: one in the 206-bp fragment and the other in the 214-bp fragment.

No such binding was observed either with the 117-bp fragment or with the 97-bp fragment (Fig. 6A). These observations suggested that the *BalI* cleavage abolished binding and that the ATP-Mg^{2+}-dependent protein bound to the region covering the *BalI* cleavage site. As described above, the ATP-Mg^{2+}-dependent protein had a second binding site in the 206-bp fragment. We therefore searched for a nucleotide sequence in the 206-bp fragment showing similarity to that covering the *BalI* site and found great similarity between the nucleotide sequences of the 206- and 214-bp fragments over the entire length (Fig. 7). This suggests the possibility that the ATP-Mg^{2+}-dependent protein recognizes a specific nucleotide sequence, at least the sequence covering the *BalI* site, and binds to this region. Another possibility is that this protein recognizes a DNA curvature, because both DNA regions show anomalous electrophoretic mobilities similar to those of the so-called bent DNA molecules, as described below.

(ii) A protein able to bind to the 97-bp fragment. The retardation of band *b* in Fig. 5 was enhanced by 5 mM $MgCl_2$. No effect of nucleotides or other cation-yielding compounds on this binding was observed. When smaller probes of the 117-bp *TaqI-BalI* fragment and the 97-bp *BalI-TaqI* fragment were used, a retarded band whose binding was enhanced by 5 mM $MgCl_2$ was detected with the 97-bp fragment (Fig. 6B) but not with the 117-bp fragment (Fig. 6A and B). The protein responsible for band *b* was therefore determined to bind to the 97-bp fragment. This protein is schematically shown as an oval in Fig. 10.

Slow mobility on gel electrophoresis of the region upstream of the *strR* promoter. During our experiments to recheck the sizes of the fragments on pTMA115 and pTMA114 by agarose and polyacrylamide gel electrophoresis, we noticed that this DNA region behaved curiously on electrophoresis. As shown in Fig. 8, the restriction fragments of the *strR* upstream region (637, 214, and 206 bp) yielded extra bands in addition to the bands with normal mobilities. The 637-bp fragment gave two distinct bands with slower mobilities when electrophoresed at 4°C. The tendency was very apparent when the DNAs were dissolved in water rather than in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA buffer, after purification of the DNAs with a Geneclean kit. The period of storage in water gave no significant difference in the proportion of the bands with slow mobilities.

Bent DNAs are known to show slow mobilities on electrophoresis (13, 16). An increased temperature during gel electrophoresis causes the mobility of a bent DNA to revert to more normal values (13, 24). A typical example is the 612-bp fragment of φX174 DNA shown in Fig. 8, lanes M. A static bend in DNA molecules exhibiting an anomalous electrophoretic mobility is formed because of local structural polymorphism in regions of homopolymeric (dA · dT) tracts consisting of at least 4 bp and appearing periodically (10). Although only three such regions (−455-AAAA, −207-TTTT, and −64-TTTT, with respect to the transcriptional start point of *strR*) are present in the 637-bp *Bam*HI-*Hin*dIII fragment, there are several blocks consisting of only A and T: −625-AAAT, −530-ATTT, −425-AATT, −291-TTAT, −222-TATT, −201-TTTATTTT, −180-AAAT, −75-TAT

TT, −44-TTTA, and −9-TATT. The frequent occurrence of these blocks is rather rare in *Streptomyces* DNA, since it has a G+C composition of 70 to 73%, and may be associated with the anomalous electrophoretic mobility of the fragments.

Determination of the transcriptional start point of the *strD* gene. Opposite transcription toward the *strD* gene encoding a putative hexose nucleotidylating enzyme was expected to start within this region, since Distler et al. (2) predicted the translational initiation codon, ATG, of the *strD* gene to be at nt −352 to −354, as shown in Fig. 1. We therefore mapped the transcriptional start point by primer extension with a 40-mer synthetic nucleotide complementary to a part of the *strD*-coding sequence. As shown in Fig. 9, a single start point was determined to be the G residue locating 197 nt upstream from the predicted ATG start codon. An additional weak signal visible on the autoradiogram is likely to be an artifact caused by premature termination of reverse transcriptase in the region of the template rich in secondary structures. There is no −35 or −10 sequence similar to typical prokaryotic promoters, 5′-TTGACA-3′ and 5′-TATAAT-3′, respectively (19, 22), in front of the start point. As for the *strR* promoter, Distler et al. (2) proposed that TTGCCC (nt −32 to −27) and TACTAT (nt −12 to −7) served as −35 and −10 sequences, respectively.

DISCUSSION

Our search for a putative transcriptional activator protein responsible for the controlled expression of *strR* by A-factor (25) has led to the identification of a protein by gel mobility retardation analyses in the present study. The retardation occurs only with the cell extract from mycelia grown in the presence of A-factor. According to our model for streptomycin biosynthesis in the A-factor regulatory relay (6, 7, 25) as well as the work by Distler et al. (2, 3), the A-factor signal is first received by the A-factor receptor protein concerned with both secondary metabolism and morphogenesis and then transmitted to the putative transcriptional activator required for the expression of *strR* on the regulatory relay unique to streptomycin production. We assume that there is one or more regulatory steps responsible for transmitting the A-factor signal from the A-factor receptor to the binding protein. Our previous study (25) suggests that the binding protein is concerned with only streptomycin production and not aerial mycelium formation. On the other hand, the combination of the A-factor receptor protein and A-factor controls both streptomycin production and aerial mycelium formation (14, 15). At any rate, we believe that further characterization of the A-factor-responsive binding protein will be a useful step to elucidate the complex A-factor regulatory cascade.

The binding site of the A-factor-responsive protein falls in the 52-bp fragment located −293 to −242 bp upstream from the transcriptional start point of *strR*, which was previously shown to function as an UAS for the A-factor-dependent promoter (25). Regarding the binding positions, we also have to point out a correction of our previous conclusion. We reported that the DNA fragment on pTMA61, which contained an internal deletion at both sides of the *Bal*I site (nt −188) in the 637-bp *Bam*HI-*Hin*dIII fragment, still showed A-factor dependence but with a greatly reduced promoter activity. Our mistakes in calculating the fragment sizes on pTMA-series plasmids prompted us to determine the exact ends of the deletion by direct nucleotide sequencing. The results showed that the DNA fragment on pTMA61 had a deletion from nt −281 to −88, the region of which covers the binding sites of most of the binding proteins detected in the present study. Although we previously judged the response of pTMA61 to A-factor to be positive, our reexamination of its A-factor dependence did not give a clear conclusion; the fragment on pTMA61 showed a higher promoter activity in the presence of A-factor in one experiment but not in the other experiment, probably because of too-low promoter activity of the DNA fragment on pTMA61 even in the presence of A-factor. It is therefore reasonable to conclude that the DNA fragment on pTMA61 shows no A-factor dependence.

During the experiments for detection of the A-factor-responsive protein, we found that three additional proteins produced in the presence and absence of A-factor bound specifically to the region near the UAS, as summarized in

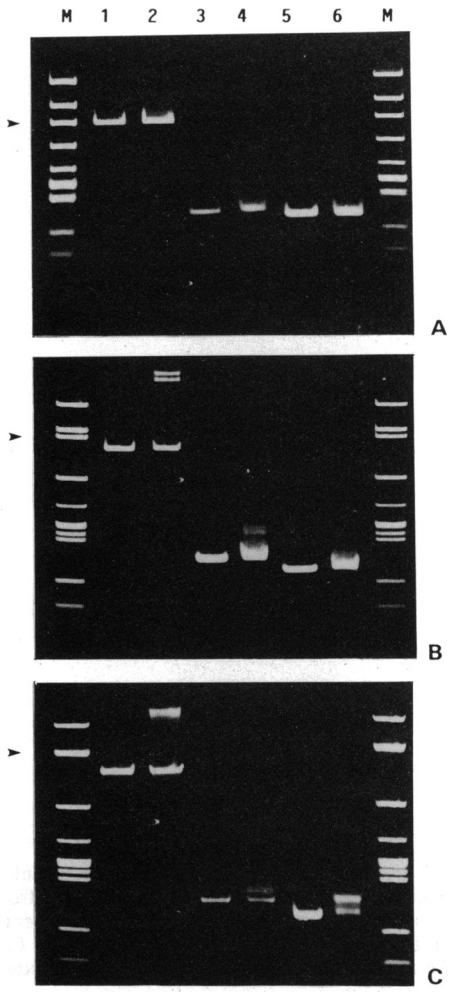


FIG. 8. Mobility analysis of restriction fragments dissected from the upstream region of the *strR* promoter. The restriction fragments analyzed were the 637-bp *Bam*HI-*Hin*dIII fragment (lanes 1 and 2), the 214-bp *Taq*I fragment (lanes 3 and 4), and the 206-bp *Taq*I fragment (lanes 5 and 6). Each restriction fragment was recovered by polyacrylamide gel electrophoresis, stored at 4°C in Tris-HCl (pH 7.2)–1 mM EDTA buffer (lanes 1, 3, and 5) and water (lanes 2, 4, and 6), and run on a 6% polyacrylamide gel at 60°C (A), room temperature (B), and 4°C (C). *Hin*cII digests of ϕX174 DNA was run in lane M. The third-largest 612-bp *Hin*cII fragment of ϕX174, indicated by arrowheads, is a typical bent DNA molecule.

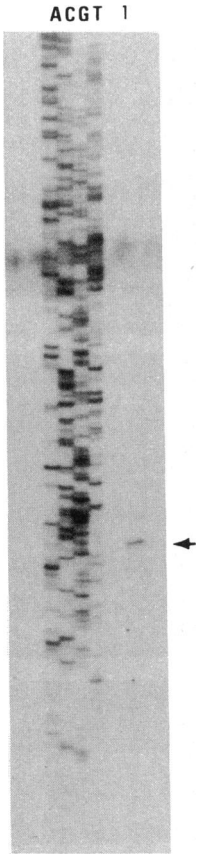


FIG. 9. Identification of the transcriptional start point of the *strD* gene by primer extension analysis. RNA was isolated from a 4-day-old (lane 1) culture of *S. griseus* IFO 13350. The sequence ladders, derived from the same primer, are shown with the A, C, G, and T reactions. An arrow indicates products of the primer extensions.

Fig. 10. Although their precise functions are still unknown, we speculate that one or more of them play some regulatory role in modulating binding of the A-factor-dependent protein to the UAS and its activity. It is especially interesting that one of them binds to both sides of the UAS and that its binding is abolished in the presence of ATP and Mg^{2+}. The investigation of how the A-factor-responsive protein in conjunction with these binding proteins controls the transcription of the *strR* promoter obviously requires the purification of each protein. Further work will reveal a possible interaction between the A-factor-responsive binding protein and RNA polymerase as well as sigma factors which probably bind to the −35 and −10 sequences contained in the 68-bp *Taq*I-*Hin*dIII fragment.

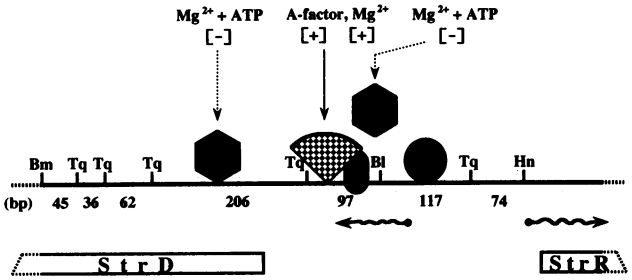


FIG. 10. Binding proteins able to bind specifically to the upstream region of the *strR* promoter. The arrows below the restriction map indicate transcriptional start points and their directions. Presumptive coding regions for *strR* and *strD* are also shown by bars. The A-factor-responsive binding protein, shown by the checked wedge, binds to the region from −293 to −242. The protein shown by a hexagon binds at the two different positions, and its binding was abolished in the presence of both ATP and Mg^{2+}. Two other proteins, shown by an oval and a circle, bind to the 97-bp *Taq*I-*Bal*I fragment and 117-bp *Bal*I-*Taq*I fragment, respectively.

The region bound by the above-mentioned proteins was found to be transcribed in an orientation opposite to that of the *strR* transcript. Unlike that of the *strR* promoter, the transcription of *strD* was not directly affected by A-factor when examined with the promoter-probe vector pTMA1 and the 637-bp *Hin*dIII-*Bam*HI fragment in the correct orientation, and the basal promoter activity in the absence of A-factor was lower than those of *strR* and *strB* (unpublished observation). This means that the A-factor-dependent protein able to activate the *strR* promoter is unable to activate the *strD* promoter. An explanation is that the binding position or orientation with respect to the promoter is important for the A-factor-responsive binding protein to serve as a transcriptional activator. On the other hand, the A-factor-responsive protein and the other binding proteins are supposed not to interfere significantly with the elongation of *strD* transcript, in some unknown way, under the circumstance in which the streptomycin biosynthetic genes, including *strD*, are actively transcribed and streptomycin is steadily produced.

The DNA region bound by the above-mentioned proteins behaves like a bent DNA molecule on agarose and polyacrylamide gel electrophoresis. A slow electrophoretic mobility of a DNA fragment is a hallmark of bent DNA (13, 16). Short DNA segments that were randomly cloned from *Streptomyces* total DNA as the fragments exhibiting slow electrophoretic mobilities contain several homopolymeric (dA · dT) stretches (24), just like the region upstream of the *strR* promoter. Each of the 206- and 214-bp fragments contains a bending region, because each of them shows a slow electrophoretic mobility. It is possible that the bent DNA structure is one of the important factors for these proteins to recognize and bind to their own binding sites. There have been observations to support the idea that DNA bending is an important feature of sites recognized by specific proteins that are involved in gene expression and initiation of DNA replication (11, 16, 21, 23). For example, the *ompF* promoter contains a bending region to which a transcriptional activator protein, OmpR, binds (16). Binding of the A-factor-dependent protein to the UAS of the *strR* gene is analogous to these examples.

ACKNOWLEDGMENTS

This work was supported in part by the Waksman Foundation of Japan and the Ministry of Agriculture, Forestry and Fisheries of Japan (BMP93-III-2-2). D. Vujaklija was supported by the Japan Society for the Promotion of Science.

REFERENCES

1. **Chodosh, L. A.** 1988. Mobility shift DNA-binding assay using gel electrophoresis, p. 2.1–2.10. *In* F. M. Ausubel et al. (ed.), Current protocols in molecular biology, vol. 1. John Wiley & Sons, New York.
2. **Distler, J., A. Ebert, K. Mansouri, K. Pissowotzki, M. Stockmann, and W. Piepersberg.** 1987. Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: nucleotide sequence of

three genes and analysis of transcriptional activity. Nucleic Acids Res. **15**:8041–8056.
3. **Distler, J., K. Mansouri, G. Mayer, M. Stockmann, and W. Piepersberg.** 1992. Streptomycin biosynthesis and its regulation in streptomycetes. Gene **115**:105–111.
4. **Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. **112**:295–298.
5. **Hara, O., and T. Beppu.** 1982. Mutants blocked in streptomycin production in *Streptomyces griseus*—the role of A-factor. J. Antibiot. **35**:349–358.
6. **Horinouchi, S., and T. Beppu.** 1992. Autoregulatory factors and communication in actinomycetes. Annu. Rev. Microbiol. **46**: 377–398.
7. **Horinouchi, S., and T. Beppu.** 1992. Regulation of secondary metabolism and cell differentiation in *Streptomyces*: A-factor as a microbial hormone and the AfsR protein as a component of a two-component regulatory system. Gene **115**:167–172.
8. **Horinouchi, S., Y. Kumada, and T. Beppu.** 1984. Unstable genetic determinant of A-factor biosynthesis in streptomycin-producing organisms: cloning and characterization. J. Bacteriol. **158**:481–487.
9. **Khokhlov, A. S., I. I. Tovarova, L. N. Borisova, S. A. Pliner, L. A. Schevchenko, E. Y. Kornitskaya, N. S. Ivkina, and I. A. Rapoport.** 1967. A-factor responsible for the biosynthesis of streptomycin by a mutant strain of *Actinomyces streptomycini*. Dokl. Akad. Nauk SSSR **177**:232–235.
10. **Koo, H.-S., H.-M. Wu, and K. M. Crothers.** 1986. DNA bending at adenine thymine tracts. Nature (London) **320**:501–506.
11. **Linial, M., and J. Shlomai.** 1987. Sequence-directed bent DNA helix in the specific binding site for *Crithidia fasciculata* nicking enzyme. Proc. Natl. Acad. Sci. USA **84**:8205–8209.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Marini, J. C., P. N. Effron, T. C. Goodman, C. K. Singleton, R. D. Wells, R. M. Wartell, and P. T. Englund.** 1984. Physical characterization of a kinetoplast DNA fragment with unusual properties. J. Biol. Chem. **259**:8974–8979.
14. **Miyake, K., S. Horinouchi, M. Yoshida, N. Chiba, K. Mori, N. Nogawa, N. Morikawa, and T. Beppu.** 1989. Detection and properties of A-factor-binding protein from *Streptomyces griseus*. J. Bacteriol. **171**:4298–4302.
15. **Miyake, K., T. Kuzuyama, S. Horinouchi, and T. Beppu.** 1990. The A-factor-binding protein of *Streptomyces griseus* negatively controls streptomycin production and sporulation. J. Bacteriol. **172**:3003–3008.
16. **Mizuno, T.** 1987. Static bend of DNA helix at the activator recognition site of the *ompF* promoter in *Escherichia coli*. Gene **54**:57–64.
17. **Mori, K.** 1983. Revision of the absolute configuration of A-factor. The inducer of streptomycin biosynthesis, basing on the reconfirmed (*R*)-configuration of (+)-paraconic acid. Tetrahedron **39**:3107–3109.
18. **Mori, K., and K. Yamane.** 1982. Synthesis of optically active forms of A-factor, the inducer of streptomycin biosynthesis in inactive *Streptomyces griseus*. Tetrahedron **38**:2919–2921.
19. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–353.
20. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
21. **Snyder, M., A. R. Buchman, and R. W. Davis.** 1986. Bent DNA at a yeast autonomously replicating sequence. Nature (London) **324**:87–89.
22. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res. **20**:969–974.
23. **Travers, A.** 1989. DNA structure: curves with a function. Nature (London) **341**:184–185.
24. **Ueki, N., H. Kinashi, and T. Mizuno.** 1991. Primary characterization of curved DNA segments cloned from *Streptomyces* DNA with extremely high G/C-contents. Agric. Biol. Chem. **55**:173–180.
25. **Vujaklija, D., K. Ueda, S.-K. Hong, T. Beppu, and S. Horinouchi.** 1991. Identification of an A-factor-dependent promoter in the streptomycin biosynthetic gene cluster of *Streptomyces griseus*. Mol. Gen. Genet. **229**:119–128.
26. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.