APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1991, p. 1386–1393
0099-2240/91/051386-08$02.00/0
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Vol. 57, No. 5

Cloning, Nucleotide Sequence, and Transcriptional Analysis of the NAD(P)-Dependent Cholesterol Dehydrogenase Gene from a *Nocardia* sp. and Its Hyperexpression in *Streptomyces* spp.

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Received 14 December 1990/Accepted 4 March 1991

NAD(P)-dependent cholesterol dehydrogenases [NAD(P)-CDH], which allow easier quantification of cholesterol by means of directly measuring the A_{340} of NAD(P)H, are useful for clinical purposes. The amino acid sequences of the NH_2 terminus and the fragments obtained by CNBr decomposition of the NAD(P)-CDH from a *Nocardia* sp. were determined for preparation of synthetic oligonucleotides as hybridization probes. A 4.4-kbp *Bam*HI fragment hybridizing to these probes was cloned on pUC19 in *Escherichia coli*. The nucleotide sequence together with the determined amino acid sequences revealed that this enzyme consists of 364 amino acids (M_r, 39,792) and contains an NAD(P)-binding consensus sequence at its NH_2-terminal portion. High-resolution S1 nuclease mapping suggested that in NAD(P)-CDH of both *Nocardia* and *Streptomyces* spp. transcription initiates at the adenine residue, which is the first position of the translational initiation triplet (AUG) of this protein. The S1 mapping experiments also showed that cholesterol-dependent regulation in the *Nocardia* sp. occurred at the level of transcription. In *Streptomyces lividans* containing the cloned fragment, however, this promoter was expressed constitutively. DNA manipulation of the cloned gene in *E. coli*, including the generation of a ribosome-binding sequence at an appropriate position by oligonucleotide-directed mutagenesis, led to production of this protein in a very large amount but in the enzymatically inactive form of inclusion bodies. On the other hand, a *Streptomyces* host-vector system was successfully used for producing 40 times as much enzymatically active NAD(P)-CDH as that produced by the original *Nocardia* sp.

Nocardia sp. strain Ch 2-1 (2) was discovered during a wide screening test for microorganisms that produce an NAD- or NADP-dependent cholesterol dehydrogenase [NAD(P)-CDH]. The enzyme produced by this strain specifically oxidizes the 3β-OH group of cholesterol with a high substrate specificity (cholesterol + $NAD^+ \rightarrow$ cholestenone + NADH + H^+) and is successfully used as a diagnostic enzyme for measurement of cholesterol in blood (1). The colorimetric assay of cholesterol with a cholesterol oxidase is often affected by bilirubin and ascorbic acid in blood. The method with NAD(P)-CDH overcomes this problem and allows easier and direct quantification of cholesterol by measuring the A_{340} of NADH as an index.

One of the purposes of this study was to improve the yield of NAD(P)-CDH by using recombinant DNA techniques, since the *Nocardia* sp. produces only a small amount of this enzyme and since control of the culture conditions which determine maximum yield of the enzyme is rather difficult. Our first attempt to produce the enzyme in *Escherichia coli* through DNA manipulation of the cloned gene was not successful; a very large amount of the NAD(P)-CDH protein produced in *E. coli* was enzymatically inactive because of the formation of inclusion bodies. However, we have succeeded in producing the active enzyme in large amounts by using a *Streptomyces* host-vector system.

Another purpose of this study was to analyze the cholesterol-inducible promoter of this gene. In the *Nocardia* sp., no detectable NAD(P)-CDH is produced in the absence of cholesterol. We have determined the nucleotide sequence and transcriptional start point of the promoter and have found that the induction by cholesterol occurs through transcriptional activation. On the other hand, this promoter is shown to be constitutively expressed in *Streptomyces* spp. The S1 mapping also revealed an unusual transcription-translation feature of this gene: the transcriptional start point is the first codon position of the translation initiation triplet AUG.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *Nocardia* sp. strain Ch 2-1 producing NAD(P)-CDH (1, 2) was provided by Amano Pharmaceutical Co. Ltd., Nagoya, Japan. *E. coli* JM109 [Δ(*lac pro*) *thi-1 endA1 gyrA96 hsdR17 relA1 recA1* F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*] (29) was used as the host for initial cloning and expression of the cloned gene and for phage M13 propagation. *Streptomyces lividans* TK24 (*str-6* SLP2$^-$ SLP3$^-$), obtained from D. A. Hopwood (11), John Innes Institute, Norwich, United Kingdom, was used as the host for manipulation and expression of the cloned gene. *Streptomyces griseus* IFO 13350 was also used for expression of the *Nocardia* gene. Ampicillin resistance plasmid pUC19 (29) containing the *lac* promoter was used as the expression plasmid in *E. coli*. As a vector plasmid for the *Streptomyces* spp., pIJ385 (thiostrepton and neomycin resistance [10]) and pIJ486 (thiostrepton resistance [27]) were used. A promoter-probe plasmid pARC1 (thiostrepton resistance [12]) was used for detecting the promoters. Phage M13 derivatives, M13mp18 and M13mp19 (29), were purchased from Amersham International.

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, and other DNA-modifying enzymes were purchased from Takara Shuzo, Co., Ltd, or Boehringer-Mannheim GmbH. *Achromobacter* peptidase was from Wako Pure Chemicals, Osaka, Japan. [α-^{32}P]dATP (3,000 Ci/

mmol) and [γ-^{32}P]ATP (5,000 Ci/mmol) were from Amersham. Thiostrepton was a gift from Asahi Chemical Industry, Shizuoka, Japan.

Determination of amino acid sequences of NAD(P)-CDH. NAD(P)-CDH was purified by a previously described method (2). Briefly, cells of *Nocardia* sp. strain Ch 2-1 were disrupted with glass beads, and the cell extract was obtained by centrifugation. Proteins were precipitated with ammonium sulfate (35% saturation). NAD(P)-CDH was further purified by successive chromatography on DEAE-cellulose and hexyl-Sepharose. For NH_2-terminal amino acid sequence determination by automated Edman degradation, an Applied Biosystems 470A sequencer was used. CNBr-decomposed polypeptides that had been separated by high-performance liquid chromatography after a standard CNBr treatment of the enzyme were similarly sequenced.

Synthesis of oligonucleotide probes. Two NAD(P)-CDH-specific oligonucleotides designed from the protein sequence data including its NH_2-terminal sequence were synthesized by the phosphoamidite method with an Applied Biosystems 380B DNA synthesizer. The oligonucleotides were purified by 15% polyacrylamide gel electrophoresis, labeled at their 5′ ends with T4 polynucleotide kinase and [γ-^{32}P]ATP, and used as hybridization probes.

Oligonucleotide screening of the NAD(P)-CDH gene. General techniques including transformation of *E. coli* cells, plasmid DNA isolation, and agarose gel electrophoresis were described by Maniatis et al. (18). Chromosomal DNA of the *Nocardia* sp. was prepared after lysis of cells with lysozyme and *Achromobacter* peptidase followed by CsCl ultracentrifugation. Southern hybridization between *Bam*HI-digested chromosomal DNA and the ^{32}P-labeled oligonucleotide probes described above was carried out according to the method of Southern (26) with the following modifications. The temperature for hybridization in 0.9 M NaCl–6 mM EDTA–90 mM Tris HCl (pH 7.5)–0.5% Nonidet P-40 (Sigma) was 25°C, and the nitrocellulose blot was washed twice at 45°C. A distinct 4.0-kb band hybridizing to the two probes was detected. From an agarose gel slice containing *Bam*HI fragments in the size range of 3.8 to 4.5 kb, DNA was recovered by the sodium perchlorate method (6) and ligated with linear molecules of pUC19 which had been successively treated with *Bam*HI and bacterial alkaline phosphatase. The ligated mixture was introduced by transformation into *E. coli* JM109, and ampicillin-resistant and nonblue transformants were selected on L broth (20) containing 50 μg of ampicillin per ml and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Colony hybridization (8) with one (probe I) of the two probes yielded one transformant showing positive hybridization. Plasmid DNA was prepared from the positive clone by CsCl-ethidium bromide ultracentrifugation. Restriction endonuclease analysis together with Southern hybridization experiments identified the region of the NAD(P)-CDH gene.

Recombinant DNA work with *Streptomyces* spp., including protoplast transformation and plasmid DNA isolation, was as described by Hopwood et al. (10).

Detection of the promoter and S1 nuclease mapping. For detection of promoter signals of the NAD(P)-CDH gene, a *Streptomyces* promoter-probe vector that allows chromogenic identification, pARC1, was used. Four restriction fragments, *Sma*I-*Ava*I (404 bp), *Pst*I-*Sma*I (380 bp), *Sal*I-*Pst*I (300 bp), and *Bam*HI-*Sal*I (400 bp), all of which are located upstream of the ATG translational start codon, were treated with the Klenow fragment of DNA polymerase I to change the ends into flush ends, and then 8-mer *Bam*HI linkers were attached to the ends. After *Bam*HI treatments of the mixtures, each of the four fragments was introduced into the *Bam*HI site upstream of the brown pigment-production genes on pARC1. Pigment production by *S. lividans* TK24 carrying these plasmids was tested on Bennett agar medium containing 30 μg of thiostrepton per ml, as previously described (12).

For high-resolution S1 protection mapping, total cellular RNAs from exponentially growing *Nocardia* sp. strain Ch 2-1 and *S. lividans* harboring pCD5 in the presence and absence of 5 mg of cholesterol per ml were prepared by using lysozyme-sodium dodecyl sulfate (SDS) and hot phenol as previously described (13). For making a ^{32}P-labeled DNA probe, a 1,350-bp *Sph*I-*Nar*I fragment covering the 404-bp *Sma*I-*Ava*I region which was shown to contain a promoter activity was end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The fragment was then cut with *Sma*I and electrophoresed on a 5% polyacrylamide gel. A *Sma*I-*Nar*I fragment of about 300 bp, obtained by the method described above, contained ^{32}P only at the 5′ end of the *Nar*I end. The DNA probe was hybridized with 30 and 15 μg of the total RNAs from the *Nocardia* sp. and *S. lividans*, respectively, in a sealed glass capillary containing 80% formamide, 0.4 M NaCl, 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4), and 1 mM EDTA (total volume, 30 μl) at 60°C for 3 h. The hybridization temperature was lowered stepwise to 42°C by 3°C per 3 h. The hybridization mixture was then diluted with 200 μl of a standard S1 buffer and digested with 300 U of S1 nuclease at 30°C for 30 min. After ethanol precipitation, the S1-protected fragments were analyzed in parallel with the sequencing ladders (19). The sequencing gel contained 8% polyacrylamide and 7 M urea.

Site-directed mutagenesis. For generation of a ribosome-binding sequence in front of the ATG translational start codon, a nucleotide 31 bp in length (5′-TTTCAGTTAAGCT TGGAGGTGCCCGTCATGG-3′) was synthesized with the DNA synthesizer. As the target DNA, the 950-bp *Sma*I-*Sma*I fragment covering this region was subcloned into the polylinker of phage M13mp19. Site-directed mutagenesis was carried out essentially by the method of Zoller and Smith (30) throughout. The mutated DNA fragment was transferred to the original plasmid, pCD1.

NAD(P)-CDH assay. The NAD(P)-CDH activity was assayed by measuring the increase in A_{340} of NADH at 30°C. The assay mixture (total volume, 3.1 ml) contained 1.67 mM cholesterol, 1.35 mM β-NAD, 100 mM Tris HCl (pH 8.5), 0.05% Triton X-100, and 0.1 ml of enzyme preparations. Cholesterol was first dissolved in 2-propanol and then diluted with a 2% Triton X-100 solution. One unit of enzyme was defined as the amount catalyzing reduction of 1 μmol of NADH per min. Proteins were quantified by using a Bio-Rad protein assay kit with bovine serum albumin as the standard.

For preparation of the crude extract from *E. coli* cells, *E. coli* JM109 containing the NAD(P)-CDH gene on pUC19 was grown overnight at 30°C in 10 ml of L broth containing 50 μg of ampicillin per ml. The culture was diluted 1:100 into the fresh medium and further incubated at 30°C for 2 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added at a final concentration of 2 mM for the induction of the *lac* promoter, and the incubation was continued for various periods. The cells were harvested by centrifugation and washed once with 50 mM Tris HCl, pH 7.5. After sonication of the washed cells, the sonicate was centrifuged at 2,000 × *g* for 10 min to separate the soluble and insoluble fractions. Preliminary experiments had shown that the NAD(P)-CDH enzyme was produced in the form of inclusion bodies, and

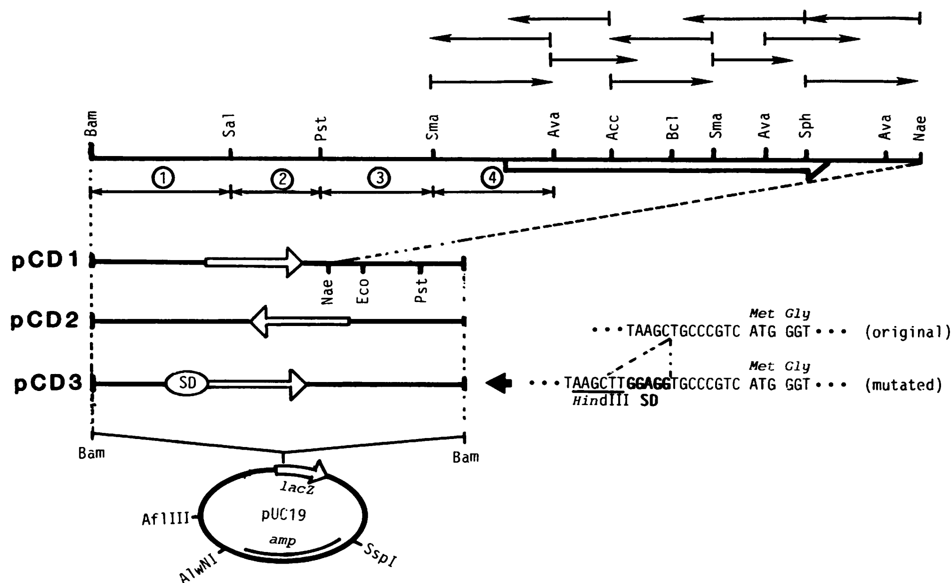

FIG. 1. Structures of the original cloned plasmid, pCD1, together with its derivative plasmids and strategy for sequencing the NAD(P)-CDH gene. The open arrows indicate the coding region of NAD(P)-CDH. Plasmid pCD2 contains the 4.4-kb fragment in an orientation opposite to that in pCD1. For construction of pCD3, an SD sequence, GGAGG, was generated 8 bp upstream from the ATG start codon by oligonucleotide-directed mutagenesis, as shown. Circled numbers 1 to 4 below the enlarged restriction map show the restriction fragments which were tested for a promoter activity by using the promoter-probe vector pARC1.

therefore we used the low-speed centrifugation which is usually employed for collecting inclusion bodies. Each fraction was assayed for NAD(P)-CDH activity by the method described above and also by 0.1% SDS–10.0% polyacrylamide gel electrophoresis (17). Protein bands were visualized by staining with Coomassie brilliant blue.

For the assay of NAD(P)-CDH activity in the cell extract from the *Streptomyces* spp., the strains containing plasmids were grown at 30°C for 2 days in medium containing the following (per liter): glucose, 10 g; Meast P1G (Asahi Beer Co.), 5 g; cultivater (fish meat extract; Yaizu-Suisan Co.), 10 g; Snow-peptone (Yukijirushi), 5 g; K_2HPO_4, 3.5 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; Adekanol (defoamer), 1 drop; and 5 μg of thiostrepton per ml. Three milliliters of the culture was transferred to 100 ml of the same medium and incubated at 30°C on a reciprocal shaker. The mycelium was collected by filtration with Toyo filter paper no. 2 and disrupted by grinding with Al_2O_3 (five-times-greater mass than that of the mycelium) in a mortar. To the disrupted mycelium, 5 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100 was added. The supernatant was used for the enzyme assay after centrifugation at 12,000 × *g* for 30 min.

Nucleotide sequence accession number. The nucleotide sequence of the NAD(P)-CDH gene has been submitted in the DDBJ, EMBL, and GenBank Nucleotide Sequence Data bases with the accession number D90244.

RESULTS

Cloning of the NAD(P)-CDH gene. NAD(P)-CDH was purified from *Nocardia* sp. strain Ch 2-1, and its NH_2-terminal amino acid sequence along with the partial amino acid sequences for two CNBr-decomposed fragments was determined. On the basis of these amino acid sequences, two 32-mer oligonucleotides in which inosines are used for all the bases corresponding to the third codon positions were synthesized, as described in Materials and Methods. For the NH_2-terminal sequence, Gly-Asp-Ala-Ser-Leu-Thr-Thr-Asp-Leu-Gly-Cys-Val-Leu-Val-Thr-Gly-X-Ser-Gly-Phe (where X stands for an amino acid which was not unambiguously determined), a 32-mer nucleotide sequence (probe I) corresponding to the amino acids from the 6th Thr to the 16th Gly, ACI ACI GAI CTI GGI TGI GTI CTI GTI ACI GG, was synthesized. For one of the amino acid sequences determined from the CNBr-decomposed fragments, Phe-X-Lys-X-Phe-Glu-Asn-Val-Leu-Ala-Gly-His-Val-Lys-Val-Leu-Val-Gly-Asn-Lys, a 32-mer nucleotide sequence (probe II) corresponding to the amino acid sequence from the 10th Ala to the 20th Lys, GCI GGI CAI GTI AAI GTI CTI GTI GGI AAI AA, was also synthesized. The synthetic DNA (probe I) designed for the NH_2-terminal amino acid sequence was used as the hybridization probe for the initial cloning of the NAD(P)-CDH gene.

Southern blot hybridization between ^{32}P-labeled probe I and the *Nocardia* sp. chromosomal DNA digested with *Bam*HI gave more than 10 bands giving positive signals with various intensities (data not shown). We chose the band (approximately 4 kb) that showed the strongest signal for the candidate of the target gene. The *Bam*HI-digested fragments of 3.8 to 4.5 kb were purified by agarose gel electrophoresis, ligated with *Bam*HI-digested pUC19 DNA, and introduced by transformation into *E. coli* JM109. About 220 ampicillin-resistant transformants which did not turn blue on an X-Gal plate were screened by colony hybridization with probes I and II. One colony showing positive hybridization to both probes was obtained. Figure 1 shows the restriction map of the plasmid, named pCD1. Southern hybridization experiments with purified pCD1 DNA containing a 4.4-kb *Bam*HI fragment revealed that probes I and II hybridized to the same 0.95-kb *Sma*I fragment in the cloned fragment.

Nucleotide sequence of the NAD(P)-CDH gene. On the basis of the results described above, we determined the nucleotide sequence of the NAD(P)-CDH gene by the chain-terminating

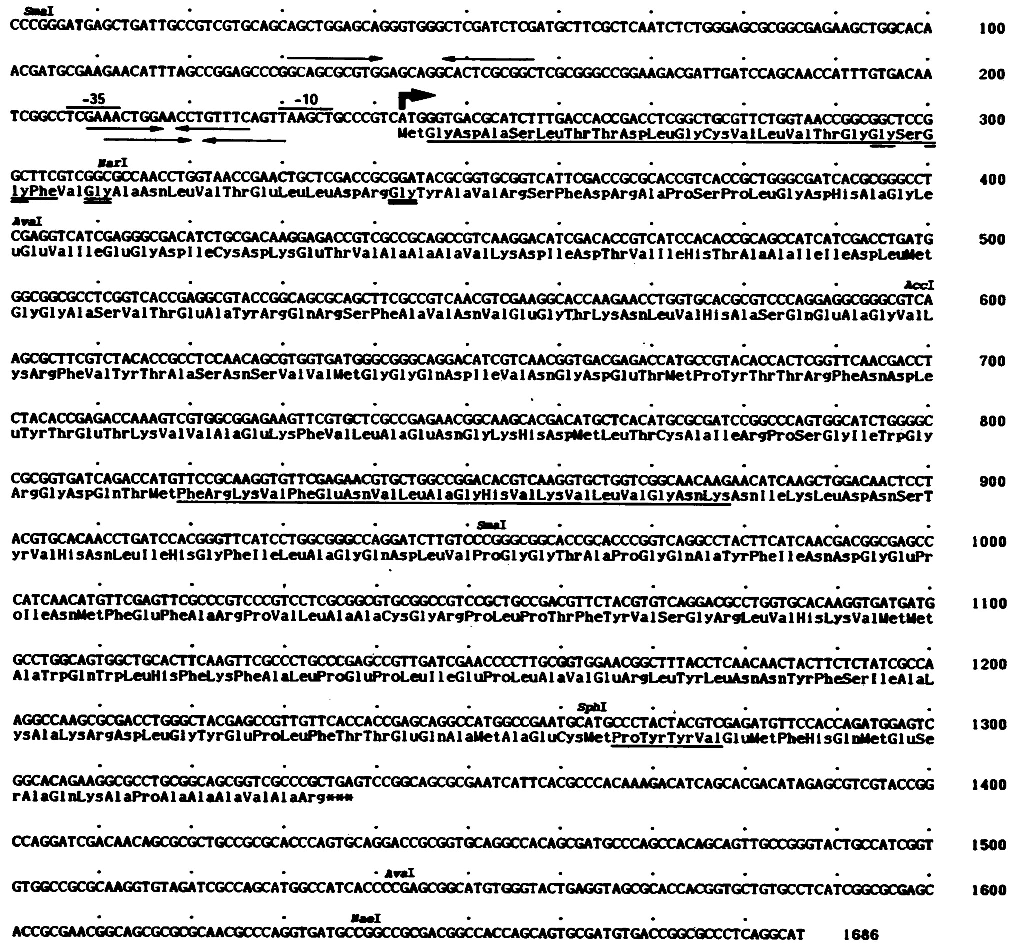

FIG. 2. Nucleotide sequence of the *Nocardia* NAD(P)-CDH gene and its deduced amino acid sequence. The underlined amino acid sequences were determined by the Edman degradation procedure, described in the legend to Fig. 1. Four Gly residues in the NAD(P)-binding consensus sequence, Gly-X-Gly-$(X)_2$-Gly-$(X)_{10}$-Gly, are indicated by double underlines. The bent arrow at nt 243 indicates the transcriptional start point, as determined by S1 nuclease mapping (see Fig. 5). The −35 and −10 sequences with some similarity to procaryotic consensus sequences are also shown. Three pairs of inverted repeat sequences upstream of the coding region are shown by opposing arrows.

dideoxynucleotide method (29), according to the strategy shown in Fig. 1. The whole nucleotide sequence was determined in both orientations, and all the restriction sites used for cloning were verified by determination as part of an overlapping sequence. Figure 2 shows the 1,686-bp nucleotide sequence and the amino acid sequence derived therefrom. An open reading frame of 364 amino acids (M_r, 39,792), which started with methionine and terminated with a TGA codon, was found to code for the amino acid sequences corresponding exactly to those determined by using the purified enzyme. The calculated size of this protein was in good agreement with that estimated from the mobility of the purified enzyme on an SDS-polyacrylamide gel. All these data clearly indicated that this open reading frame represented NAD(P)-CDH of the *Nocardia* sp.

Comparison of the NH_2-terminal sequence determined with the mature enzyme and that deduced from the nucleotide sequence suggested that the NH_2-terminal methionine was processed after translation. A search for a consensus sequence for NAD(P)-binding sites which is well conserved among the NAD-binding domains of many NAD(P)-dependent dehydrogenases (23, 28) revealed the presence of Gly-X-Gly-$(X)_2$-Gly-$(X)_{10}$-Gly (where X stands for any amino acid) at the NH_2-terminal region of this protein. Alignment of this region, called the "fingerprint" region, is shown in Fig. 3.

The overall average G+C composition of the coding region is 63.7 mol%, and those of codon positions 1, 2, and 3 are 61.9, 40.3, and 88.8 mol%, respectively. The relatively high G+C content is reflected preferentially at the third codon position. Such a codon usage pattern is characteristic for genes with a high G+C content from various bacteria (4).

Enzyme	Position	Sequence
Alcohol DH (*Drosophila*)	14	G L G G I G L D T S K Q L L K R D
Cholesterol DH (*Nocardia*)	18	G S G F V G A N L V T E L L D R G
Lactate DH (*Bacillus*)	13	G A G F V G A S Y V F A L M N Q G

FIG. 3. Amino acid comparisons of NAD(P)-binding domains. The fingerprint regions of alcohol dehydrogenase from *Drosophila melanogaster* and of a lactate dehydrogenase from *Bacillus stearothermophilus* are aligned with that of NAD(P)-CDH from the *Nocardia* sp. The Gly residues that form the β-α-β dinucleotide-binding fold (28) are underlined. The number for each sequence indicates the position of the first residue in the primary structure. For individual references, see reference 28.

Transcriptional start point and promoter region of the NAD(P)-CDH gene. For identification of promoter signals of the NAD(P)-CDH gene, we first employed a convenient *Streptomyces* host-vector system, because as described below, this gene contained a promoter functional in *Streptomyces* spp. Four restriction fragments, shown in Fig. 1, located upstream of the translational start codon were inserted into the *Bam*HI site of the promoter-probe vector pARC1 to test for promoter activities. Plasmid pARC1 directs brown pigment production in *S. lividans* when a DNA fragment containing a promoter is inserted in the *Bam*HI site in the correct orientation (12). By this analysis, only the *Sma*I-*Ava*I fragment (nucleotides [nt] 1 to 399) in only one orientation caused *S. lividans* to produce the pigment, showing the presence of a promoter(s). The transcriptional orientation was the same as that for translation of the NAD(P)-CDH enzyme. Since NAD(P)-CDH is inducibly produced by cholesterol in the original *Nocardia* strain, we examined the effect of cholesterol on pigment production in this system. However, no stimulation of pigment production by 5 mg of cholesterol per ml in the agar medium was observed. This result suggested that a promoter detected by this system was constitutively expressed in *S. lividans*, although, as will be described below, this promoter was responsible for the induction observed in the *Nocardia* sp.

For determination of the transcriptional start point by S1 mapping, we constructed plasmid pCD5 (Fig. 4), in which the NAD(P)-CDH gene was located downstream of the rho-independent transcriptional terminator in pIJ486 (27), in order to avoid possible effects caused by read-through from the vector sequence. This plasmid was also used for production of the enzyme, as shown below. High-resolution S1 mapping with total RNAs from *S. lividans* containing pCD5 which was grown in the presence and absence of cholesterol was conducted with the ^{32}P-labeled *Sma*I-*Nar*I fragment (nt 1 to 309). A transcriptional start point was determined to be nt 243 (Fig. 5). This residue, A, was the first position of the translational start ATG codon. The ^{32}P-signals for RNAs prepared in the presence and absence of cholesterol were almost the same in intensity; this is consistent with the results described above obtained by use of the promoter-probe plasmid pARC1.

On the other hand, similar experiments with RNAs prepared from the *Nocardia* sp. clearly showed that the promoter was induced by cholesterol in the *Nocardia* sp. cells. The transcriptional start points in the *Nocardia* sp. and *S. lividans* were the same. It was therefore concluded that the inducible production of NAD(P)-CDH by cholesterol resulted from the transcriptional stimulation of the gene in some unknown way.

The nucleotide sequence (5'-TCGAAA-3' for −35 and 5'-TAAGCT-3' for −10, with a 17-bp space) upstream from the transcriptional start point showed some similarity to consensus sequences (5'-TTGACA-3' for −35 and 5'-TATAAT-3' for −10, with a 17-bp space) for other procaryotic promoters (22). It also showed similarity to one type (5'-TTGACA-3' for −35 and 5'-TAGGAT-3' for −10, with a 18-bp space) of *Streptomyces* promoter, which is believed to be active during the vegetative growth (9). The NAD(P)-CDH gene is most likely expressed in *Streptomyces* spp. because the consensus sequence of its promoter sequence is similar to that of one class of *Streptomyces* promoters. *Streptomyces* spp. allow expression of most procaryotic promoters with the consensus sequences (3). The observation that NAD(P)-CDH expression is constitutive in *S. lividans* suggests that the *Nocardia* sp. CDH regulatory proteins are not present in *S. lividans*.

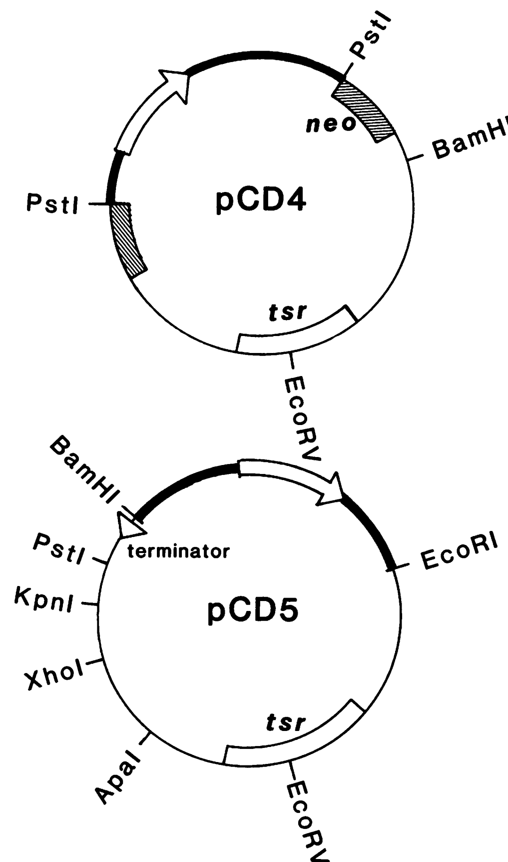

FIG. 4. Structures of pCD4 and pCD5. *tsr*, thiostrepton resistance gene; *neo*, neomycin resistance gene. For construction of pCD4, the 3.2-kb DNA fragment containing the whole NAD(P)-CDH gene (shown by an open arrow) was excised by digestion of pCD1 with *Pst*I and ligated with *Pst*I-digested pIJ385. The *Pst*I site on pIJ385 is in the *neo* gene. The ligated mixture was introduced by transformation into protoplasts of *S. lividans* TK24, and they were regenerated on R2YE medium (10). Transformants were selected by replication onto Bennett agar medium containing 30 μg of thiostrepton per ml. The plasmid pCD4, obtained in this way, contains the NAD(P)-CDH gene in the same orientation as that of transcription of the *neo* gene. Plasmid pCD5 was constructed by insertion of the 3.3-kb *Bam*HI-*Eco*RI fragment containing the whole NAD(P)-CDH gene into the multilinker site of pIJ486. The NAD(P)-CDH gene in pCD5 is located downstream of a transcriptional terminator derived from *E. coli* phage fd. This terminator was shown to prevent significant transcriptional read-through from the vector sequence (27).

Expression of the NAD(P)-CDH gene in *E. coli*. First we attempted to express the cloned gene by using *E. coli* host-vector systems. Because of the absence of a possible ribosome-binding sequence (SD [24]) at an appropriate position upstream of the translational start codon of NAD(P)-CDH, we generated a GGAGG sequence 8 bp upstream of the start codon by oligonucleotide-directed mutagenesis, as shown in Fig. 1. The NAD(P)-CDH gene with the artificial SD sequence was then placed downstream of the *lac* promoter in pUC19, resulting in pCD3. We also constructed a pCD2 plasmid in which the NAD(P)-CDH gene was placed in the opposite orientation to that in pCD1 (Fig. 1). *E. coli*

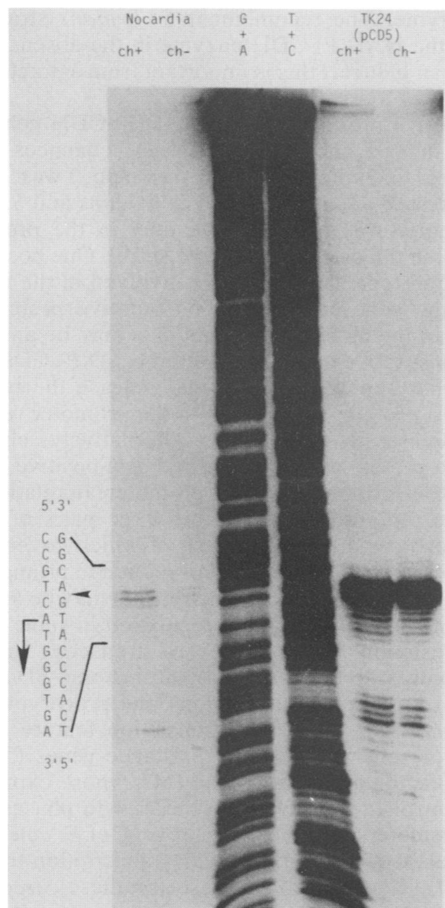

FIG. 5. Position of the 5′ terminus of the transcript directed by the promoter of the NAD(P)-CDH gene as determined by S1 nuclease mapping. Total RNAs (15 μg each) prepared from exponentially growing *S. lividans* TK24 harboring pCD5 in the presence (Ch^+) and absence (Ch^-) of cholesterol were hybridized with the ^{32}P-labeled *Sma*I-*Nar*I fragment and analyzed in parallel with the sequence ladders (lanes G+A and T+C). The arrowhead indicates the position of the S1-protected fragment with the strongest intensity. The 5′ terminus of the mRNA is assigned to the indicated position, because the fragments generated by the chemical sequencing reactions migrate 1.5 nt further than the corresponding fragments generated by S1 nuclease digestion of the RNA-DNA hybrids (half a residue from the presence of the 3′-terminal phosphate group and one residue from the elimination of the 3′-terminal nucleotide) (25). Similarly, total RNAs (30 μg each) from *Nocardia* sp. strain Ch 2-1 grown in the presence and absence of cholesterol were used for S1 mapping. The terminus of mRNA is the same as that produced in *S. lividans*. In addition, it is clear that cholesterol induces the expression of this promoter.

JM109 harboring pCD1, pCD2, or pCD3 was cultured, and the *lac* promoter was induced by the addition of IPTG. However, no NAD(P)-CDH activity was detected in either the particulate or soluble fraction from any transformant. SDS-polyacrylamide gel electrophoresis of the particulate fraction of *E. coli* harboring pCD3 showed that the NAD(P)-CDH protein was produced in a very large amount, depending on the presence of IPTG (Fig. 6). The NAD(P)-CDH protein composed about 10% of the total proteins of *E. coli* cells. In addition, to our surprise, plasmid pCD1, which we used as a reference in this experiment, also directed the

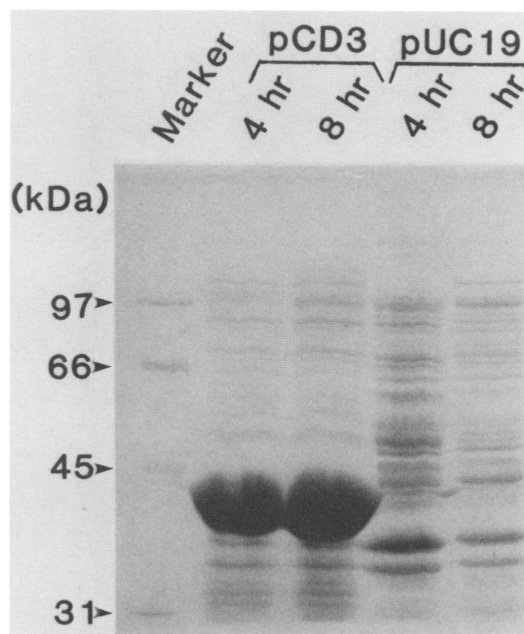

FIG. 6. Production of the NAD(P)-CDH protein in *E. coli*. *E. coli* JM109 harboring pCD3 or pUC19 was grown, and the *lac* promoter was induced by IPTG. The insoluble fractions prepared from the cells grown for 4 and 8 h in the presence of IPTG were analyzed by SDS-polyacrylamide gel electrophoresis. An NAD(P)-CDH protein of about 40 kDa was produced in a very large amount by *E. coli* harboring pCD3. At the zero time of IPTG addition, no thick protein band at this position was seen (data not shown). Molecular mass standards used are phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).

synthesis of the NAD(P)-CDH protein in almost the same amount as did pCD3 (data not shown). This implies that in *E. coli* some sequence serves as an efficient SD sequence. The most probable candidate is AAG (nt 231 to 233), which is located 9 bp upstream of the ATG start codon. Microscopic observation of IPTG-induced *E. coli* cells harboring pCD1 or pCD3 revealed an inclusion body in each cell. It seems probable that the NAD(P)-CDH protein was produced in a large amount in the inactive from of inclusion bodies.

Plasmid pCD2 failed to direct the synthesis of the NAD(P)-CDH protein, presumably because transcription of the NAD(P)-CDH gene initiates at the *lac* promoter in *E. coli* cells containing pCD1 and pCD3 and because the promoter of the NAD(P)-CDH gene is not functional in *E. coli*. As described above, the promoter signal of the NAD(P)-CDH gene has similarity to the consensus promoter signal of *E. coli*. It is well conceivable that notwithstanding its similarity, this promoter is not functional in *E. coli*. This is consistent with the observations that most promoters from *Streptomyces* spp., and probably from *Nocardia* spp., are not recognized by the *E. coli* transcriptional machinery (3, 15).

Overexpression of the NAD(P)-CDH enzyme in *Streptomyces* spp. We next tried to produce the enzymatically active NAD(P)-CDH protein in *Streptomyces* spp. which are closely related to the original *Nocardia* strain. Our preliminary experiments with *S. lividans* containing pCD4 or pCD5 and *S. griseus* containing pCD4 showed that these strains produced almost the same amounts of active NAD(P)-CDH, irrespective of the presence and absence of cholesterol. We

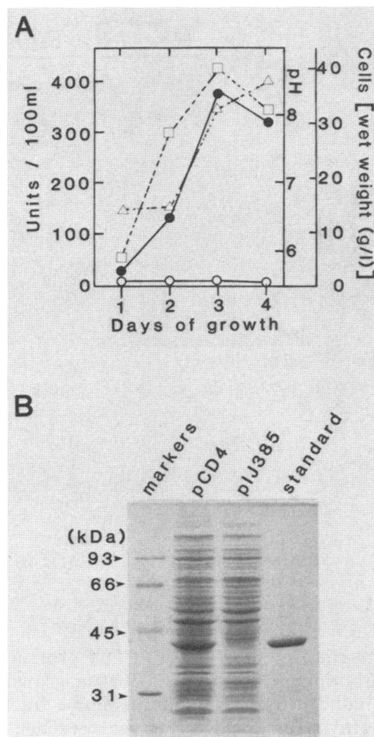

FIG. 7. Time course of NAD(P)-CDH production by *S. lividans* TK24 harboring pCD4 (A) and SDS-polyacrylamide gel electrophoresis of the soluble fraction of this strain (B). (A) The NAD(P)-CDH activities specified by *S. lividans* (pCD4) (●) together with the pH of the culture broth (△) and wet cell weight (□) were monitored. *S. lividans* (pIJ385) (○), as a control, produced no NAD(P)-CDH throughout the growth. (B) The protein profile of the soluble fraction prepared from a 3-day-old culture of *S. lividans* harboring pCD4 (lane 2) or pIJ385 (lane 3) is analyzed. Molecular mass standards and the purified NAD(P)-CDH protein are run in lane 1 and lane 4, respectively.

chose *S. lividans* containing pCD4 for detailed analysis of production of NAD(P)-CDH. As shown in Fig. 7, this strain produced 397 U of active NAD(P)-CDH per 100 ml of culture, whereas *S. lividans* containing only the vector plasmid pIJ385 produced no NAD(P)-CDH. The NAD(P)-CDH protein that accumulated in the cells was detected as a thick band on SDS-polyacrylamide gels (Fig. 7B).

The activity specified by this recombinant *S. lividans* strain was about 40 times higher than that by the original *Nocardia* sp. The maximum activity obtained with the *Nocardia* sp. amounted to only 10 to 20 U per 100 ml of culture when grown in the presence of cholesterol as an inducer, despite many efforts at improving the yield. In addition, control of the culture for stable production of the enzyme had been a problem to be solved.

DISCUSSION

DNA manipulation using *E. coli* systems often resulted in production of proteins in the inactive form of inclusion bodies, which is usually very difficult to be refolded into an active form. The NAD(P)-CDH protein of the *Nocardia* sp. was produced as inclusion bodies as well. The use of a *Streptomyces* host-vector system, however, not only overcame this difficulty but also led to a great yield enhancement of the enzyme. The recombinant *S. lividans* strain stably produced the NAD(P)-CDH enzyme in the absence of cholesterol as an inducer; this is important from a practical point of view.

The putative promoter of the NAD(P)-CDH gene having similarity to −35 and −10 consensus sequences of other procaryotes, including *Streptomyces* spp., was constitutively expressed at a high level in *S. lividans* and *S. griseus*. This promoter was highly active only in the presence of cholesterol in the original *Nocardia* strain. One possibility is that a certain repressor protein is involved in the induction and that the gene coding for this putative protein is not contained in the cloned fragment. This may be an explanation for constitutive expression of the NAD(P)-CDH gene in *Streptomyces* spp. Cholesterol may release the repression caused by a protein which binds to the promoter region and which represses its expression. Alternatively, cholesterol may bind a protein which is required as a positive regulator to initiate transcription from this promoter. In relation to this speculative repressor or activator, three pairs of inverted repeat sequences are present, two of which cover the −35 and −10 regions of the cholesterol-inducible promoter (Fig. 2). One or some of these inverted repeats may be associated with the cholesterol-dependent regulation in some way.

The translation of the leaderless transcript presents a striking contrast to the conventional ribosome-SD sequence contact in translational initiation in other procaryotes (7). In *Streptomyces* spp., the same translation feature has been reported for several antibiotic resistance genes (5, 13, 16) and the A-factor production gene (14), whose expression is strictly controlled depending on the growth phases. One of the two promoters for the repressor gene of *E. coli* bacteriophage λ also shows an unusual transcription-translation feature (21). Contrary to the case for these *Streptomyces* genes, there seems to be no reason for the *Nocardia* sp. to produce the NAD(P)-CDH protein at a certain stage of growth. In fact, the *Nocardia* sp. begins to produce NAD(P)-CDH at an early stage of growth and then continues its production (data not shown). The recombinant *S. lividans* strain containing pCD4 also appears to produce NAD(P)-CDH at an early stage of growth, since the amount of NAD(P)-CDH increases with the growth measured in terms of the cell weight (Fig. 7A). Although an extensive study is apparently required, this finding suggests that the translation of leaderless transcripts is distributed more widely than expected.

ACKNOWLEDGMENTS

We are grateful to Amano Pharmaceutical Co., Ltd., for providing us with the *Nocardia* strain and for help in purification of the enzyme and cultivation of the recombinant *S. lividans* strain. We also thank Masaharu Inouye and Makoto Nishiyama for their helpful comments during this work.

REFERENCES

1. **Akiba, T.** 1983. An enzymatic measurement of cholesterol with NAD-dependent cholesterol dehydrogenases and the reagents for the assay. Japanese patent 83-89200.
2. **Akiba, T.** 1990. The method for preparation of NAD(P)-dependent cholesterol dehydrogenases. Japanese patent 90-18064.
3. **Bibb, M. J., and S. N. Cohen.** 1982. Gene expression in *Streptomyces*: construction and application of promoter-probe plasmid vectors in *Streptomyces lividans*. Mol. Gen. Genet. **187**:265–277.
4. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identifi-

cation of protein-coding sequences. Gene **30:**157–166.
5. **Bibb, M. J., G. R. Janssen, and J. M. Ward.** 1986. Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. Gene **41:**E357–E368.
6. **Chen, C. W., and C. A. Thomas, Jr.** 1980. Recovery of DNA segments from agarose gel. Anal. Biochem. **101:**339–341.
7. **Gold, L., D. Pribnow, T. Schneider, S. Shinedling, B. S. Singer, and G. Stormo.** 1981. Translational initiation in prokaryotes. Annu. Rev. Microbiol. **35:**365–403.
8. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72:**3961–3965.
9. **Hopwood, D. A., M. J. Bibb, K. F. Chater, G. R. Janssen, F. Malpartida, and C. P. Smith.** 1986. Regulation of gene expression in antibiotic-producing *Streptomyces*, p. 251–276. *In* I. R. Booth and C. F. Higgins (ed.), Regulation of gene expression—25 years on. Cambridge University Press, Cambridge.
10. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom.
11. **Hopwood, D. A., T. Kieser, H. M. Wright, and M. J. Bibb.** 1983. Plasmids, recombination and chromosome mapping in *Streptomyces lividans* 66. J. Gen. Microbiol. **129:**2257–2269.
12. **Horinouchi, S., and T. Beppu.** 1985. Construction and application of a promoter-probe plasmid that allows chromogenic identification in *Streptomyces lividans*. J. Bacteriol. **162:**406–412.
13. **Horinouchi, S., K. Furuya, M. Nishiyama, H. Suzuki, and T. Beppu.** 1987. Nucleotide sequence of the streptothricin acetyltransferase gene from *Streptomyces lavendulae* and its expression in heterologous hosts. J. Bacteriol. **169:**1929–1937.
14. **Horinouchi, S., H. Suzuki, M. Nishiyama, and T. Beppu.** 1989. Nucleotide sequence and transcriptional analysis of the *Streptomyces griseus* gene (*afsA*) responsible for A-factor biosynthesis. J. Bacteriol. **171:**1206–1210.
15. **Horinouchi, S., T. Uozumi, and T. Beppu.** 1980. Cloning of *Streptomyces* DNA into *Escherichia coli*: absence of heterospecific gene expression of *Streptomyces* genes in *E. coli*. Agric. Biol. Chem. **44:**367–381.
16. **Janssen, G. R., J. M. Ward, and M. J. Bibb.** 1989. Unusual transcriptional and translational features of the aminoglycoside phosphotransferase gene (*aph*) from *Streptomyces fradiae*. Genes Dev. **3:**415–429.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
20. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Ptashne, M., K. Backman, M. Z. Humayun, A. Jeffrey, R. Maurer, B. Meyer, and R. T. Sauer.** 1976. Autoregulation and function of a repressor in bacteriophage lambda. Interactions of a regulatory protein with sequences in DNA mediate intricate patterns of gene regulation. Science **194:**156–161.
22. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in promotion and termination of RNA transcription. Annu. Rev. Genet. **13:**319–353.
23. **Scrutton, N. S., A. Berry, and R. N. Perham.** 1990. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. Nature (London) **343:**38–43.
24. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *E. coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:** 1342–1346.
25. **Sollner-Webb, B., and R. H. Reeder.** 1979. The nucleotide sequence of the initiation and termination sites for ribosomal RNA transcription in *X. laevis*. Cell **18:**485–499.
26. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
27. **Ward, J. M., G. R. Janssen, T. Kieser, M. J. Bibb, M. J. Buttner, and M. J. Bibb.** 1986. Construction and characterization of a series of multi-copy promoter-probe plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn*5* as indicator. Mol. Gen. Genet. **203:**468–478.
28. **Wierenga, R. K., M. C. H. De Maeyer, and W. G. J. Hol.** 1985. Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins. Biochemistry **24:**1346–1357.
29. **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.
30. **Zoller, M. J., and M. Smith.** 1983. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. **100:**468–500.