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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NAD(P)-dependent cholesterol dehydrogenases [NAD(P)-CDH], which allow easier quantification of cholesterol by means of directly measuring the A<sub>260</sub> of NAD(P)H, are useful for clinical purposes. The amino acid sequences of the NH<sub>2</sub> 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 BamHI 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<sub>r</sub>, 39,792) and contains an NAD(P)-binding consensus sequence at its NH<sub>2</sub>-terminal portion. High-resolution S1 nuclease mapping suggested that in NAD(P)-CDH of both Nocardia and Streptomyces spp. transcription initiation 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<sup>+</sup>→ cholestenone + NADH + H<sup>+</sup>) 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 ascobic acid in blood. The method with NAD(P)-CDH overcomes this problem and allows easier and direct quantification of cholesterol by measuring the A<sub>260</sub> 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.

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-l endA1 gyrA96 hsdR17 relA1 recA1 F<sup>−</sup> traD36 proAB lacZ<sup>−</sup> lacZΔM15] (29) was used as the host for initial cloning and expression of the cloned gene and for phase M13 propagation. Streptomyces lividans TK24 (str<sup>−</sup> SLP2<sup>−</sup> SLP3<sup>−</sup>) 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. 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. Phase 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-Manheim GmbH. Achromobacter peptidase was from Wako Pure Chemicals, Osaka, Japan. [α-<sup>32</sup>P]dATP (3,000 Ci/
mmol) and [γ−32P]ATP (5,000 Ci/mmol) were from Ameri-
sham. Thiostrepton was a gift from Asahi Chemical Indus-
try, 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 Nocardi a sp. strain Ch 2-1 were disrupted with glass beads, and the cell extract was obtained by centrifugation. Proteins were precipitated with ammoni-
um sulfate (35% saturation). NAD(P)-CDH was further purified by successive chromatography on DEAE-cellulose and hexyl-Sepharose. For NH₂-terminal amino acid se-
quence determination by automated Edman degradation, an
Applied Biosystems 470A sequencer was used. CNBr-de-
composed 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 NH₂-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−32P]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 Nocardi a sp. was prepared after lysis of cells with lysozyme and Achromobacter peptidease followed by CsCl ultracentrifugation. Southern hybridization between BamHI-digested chromosomal DNA and the 32P-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 BamHI 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 BamHI and bacterial alkaline
phosphatase. The ligated mixture was introduced by trans-
formation into E. coli JM109, and ampicillin-resistant and nonblue transformants were selected on L broth (20) contain-
ing 50 µg of ampicillin per ml and X-Gal (5-bromo-4-
chloro-3-indolyl-β-D-galactopyranoside). Colony hybridiza-
tion (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-ethidiu-
mem bromide ultracentrifugation. Restriction endonuclease anal-
ysis together with Southern hybridization experiments iden-
tified 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 51 nuclease mapping. For detection of promoter signals of the NAD(P)-CDH gene, a Streptomyces promoter-probe vector that allows chromo-
egene identification, pARC1, was used. Four restriction frag-
ments, Smal-Aval (404 bp), PstI-Smal (380 bp), SalI-PstI
(300 bp), and BamHI-Sall (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 BamHI linkers were
attached to the ends. After BamHI treatments of the mix-
tures, each of the four fragments was introduced into the
BamHI 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 Nocardi a 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 32P-labeled DNA probe, a 1,350-bp SpHl-NarI fragment covering the 404-bp
Smal-Aval region which was shown to contain a promoter
activity was end labeled with [γ−32P]ATP and T4 polynucleo-
tide kinase. The fragment was then cut with Smal and elec-
rophoresed on a 5% polyacrylamide gel. A Smal-NarI fragment of about 300 bp, obtained by the method described above, contained 32P only at the 5' end of the NarI end. The DNA probe was hybridized with 30 and 15 µg of the total RNAs from the Nocardi a 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 1% buffer and digested with 300 U of S1 nuclease at 30°C for 30 min. After ethanol precipitation, the S1-protected fragments were ana-
lyzed 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'-TTTCAAGTTAAGCT
TTGAAGGTCCCGTCAATGG-3') was synthesized with the
DNA synthesizer. As the target DNA, the 950-bp Smal-
Smal fragment covering this region was subcloned into the
polylinker of plasmid M13mp19. Site-directed mutagenesis
was carried out essentially by the method of Zoller and
Smith (30) throughout. The mutated DNA fragment was trans-
ferred to the plasmid PCM1.

NAD(P)-CDH assay. The NAD(P)-CDH activity was as-
sayed by measuring the increase in A340 of NADH at 30°C.

The assay mixture (total volume, 3.1 ml) contained 1.67 mM
cholerol, 1.35 mM β-NAD, 100 mM Tris HCl (pH 8.5),
0.05% Triton X-100, and 0.1 ml of enzyme preparations. Chloroel was first dissolved in 2-propanol and then di-
luted 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. Isopro-
pyl-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
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; cultivator (fish meat extract; Yaizu-Suisan Co.), 10 g; Snow-peptone (Yukijirushi), 5 g; K2HPO4, 3.5 g; KH2PO4, 1 g; MgSO4·7H2O, 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 Al2O3 (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 NH2-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 NH2-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 CTI 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-Val-Lys-Val-Lys-Val, a 32-mer nucleotide sequence (probe II) corresponding to the amino acid sequence from the 10th Ala to the 20th Lys, GCC GCI CAI GTI AAI GTI CTI GTI GGI AA, was also synthesized. The synthetic DNA (probe I) designed for the NH2-terminal amino acid sequence was used as the hybridization probe for the initial cloning of the NAD(P)-CDH gene.

Southern blot hybridization between 32P-labeled probe I and the Nocardia sp. chromosomal DNA digested with BamHI 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 BamHI-digested fragments of 3.8 to 4.5 kb were purified by agarose gel electrophoresis, ligated with BamHI-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 BamHI fragment revealed that probes I and II hybridized to the same 0.95-kb SmaI 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 from it. 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).

<table>
<thead>
<tr>
<th>Alcohol DH (Drosophila)</th>
<th>14</th>
<th>GLGCGILKDFSLKDLKRD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol DH (<em>Nocardia</em>)</td>
<td>18</td>
<td>GSGFVGMNLVTDELLDRG</td>
</tr>
<tr>
<td>Lactate DH (bacillus)</td>
<td>15</td>
<td>GAOGPGAVYFFALMNQG</td>
</tr>
</tbody>
</table>

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 steaerothermophilus* are aligned with that of NAD(P)-CDH from the *Nocardia* sp. 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 BamHI 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 BamHI site in the correct orientation (12). By this analysis, only the Smal-Aval 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 32P-labeled Smal-NarI 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 32P-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 promoters, 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.

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
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 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 possible that the NAD(P)-CDH protein was produced in a large amount in the inactive form 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
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 proaryotes, including *Streptomyces* sp., 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* sp. 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 proaryotes (7). In *Streptomyces* sp., 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.

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