An Operon Containing the Genes for Cholesterol Oxidase and a Cytochrome P-450-Like Protein from a *Streptomyces* sp.

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The nucleotide sequence of the promoter region of the gene for cholesterol oxidase (choA) from *Streptomyces* sp. strain SA-COO was determined. We found an open reading frame (choP) that is located between a potential promoter sequence and the structural gene for the ChoA protein. Deletion analysis showed that the promoter region for *choP* is essential for expression of the *choA* gene. Mappings of S1 nuclease and primer extension of transcripts generated in vivo suggested that the synthesis of mRNA starts at a site 41 bases upstream from the ATG initiation codon of the *choP* gene. By Northern (RNA) blot analysis of the transcripts, we found a 2.9-kilobase transcript that is identical in size to the total sequence of the *choP* and *choA* genes. These results suggest that the two genes, *choP* and *choA*, are transcribed polycistronically under the control of the promoter that is upstream from the structural gene for *choP*. The *choP* gene encodes a protein of 381 amino acids with a calculated M_r of 41,668. The nucleotide sequence of the *choP* gene has a high degree of similarity to the sequence of the genes for cytochrome P-450s from humans and *Pseudomonas* species. A region of homology with the cytochrome P-450s from various organisms was identified in the *choP* gene revealed a unique peak, characteristic of cytochrome P-450, which is identical to that obtained with the parent strain.

Streptomyces species produce a large variety of medically important metabolites and extracellular enzymes, in particular cholesterol oxidase (15, 17, 25). Cholesterol oxidase (EC 1.1.3.6) catalyzes the oxidation of cholesterol (5-cholesten-3-B-ol) to 4-cholesten-3-one, with the reduction of oxygen to hydrogen peroxide. The enzyme is commonly used for the enzymatic transformation of cholesterol (2, 36) and is also used for quantitation of levels of cholesterol in clinical specimens, by coupling of the enzyme with peroxidase (1). Recently, we cloned the gene involved in the production of cholesterol oxidase (choA) from Streptomyces sp. strain SA-COO (25). Cells of Streptomyces lividans carrying a plasmid that includes the choA gene overproduce extracellular cholesterol oxidase when cultured in an appropriate medium. The nucleotide sequence of the choA gene revealed that the gene of cholesterol oxidase starts from a GTG initiation codon and terminates at a TAA termination codon. In the 3'-flanking region of choA, a long palindromic sequence, which may act as a transcription terminator, was found. The gene encodes a mature cholesterol oxidase of 504 amino acids with a calculated M_r of 54,913. The leader peptide extends over 42 amino acids and has the characteristics of a signal sequence. The amino acid sequence of the N-terminal protein of the purified enzyme agrees with the data deduced from nucleotide sequencing. However, our results did not allow us to identify the promoter sequences upstream from the choA gene.

Thus, we searched for the promoter region of the choA gene and found a new open reading frame (ORF). In this report, we describe the nucleotide sequence that is upstream from the choA gene, the sites of initiation of transcription of mRNA, and an analysis of transcripts in the cho region. We

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were *Streptomyces* sp. strain SA-COO, a strain that produces extracellular cholesterol oxidase (provided by Toyobo Co. Ltd., Tsuruga, Japan) and *S. lividans* 1326, provided by D. Hopwood. pCO-3, a plasmid that contains the *Streptomyces* genes *choA* and *choP* and the thiostrepton resistance (*tsr*) gene derived from pIJ702 (19), was described previously. pCO-6, pCO-7, and pCO-51 are deletion plasmids derived from pCO-3. M13mp18 and M13mp19 (39) were obtained from Takarashuzo Co. Ltd., Kyoto, Japan.

Culture media. Liquid cultures of *Streptomyces* cells were prepared in YEME medium (5). Strains carrying pCO-3 or its derivatives were grown in the presence of 50 μ g of thiostrepton per ml.

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, reverse transcriptase, S1 nuclease, and Klenow fragment of DNA polymerase were purchased from either Toyobo Co. or Takarashuzo Co. Ltd. The M13 sequencing kit with deoxy-7-deazaguanosine triphosphate was purchased from Takarashuzo Co. Ltd. $[\alpha^{-32}P]dCTP$ (>600 Ci/mmol) and $[\gamma^{-32}P]ATP$ (4,500 Ci/mmol) were purchased from Amersham Co., Arlington Heights, Ill. Isopropyl- β -D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-3- β -galactoside were purchased from Sigma Chemical Co., St. Louis, Mo. Thiostrepton was generously provided by Asahikasei Co., Ltd., Tokyo, Japan.

Preparation of RNA. Total RNA was extracted from *S. lividans* 1326 that carried pCO-3, pCO-6, or pIJ702, grown for 1 day in YEME medium, by a modification of a procedure of Horinouchi et al. (13). Pelleted mycelia were suspended in 0.3 M sucrose-50 mM Tris hydrochloride (pH

also discuss homologies in the new ORF and the finding of a predicted cytochrome P-450 from *Streptomyces* species.

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FIG. 1. Restriction maps of pCO-3 and derivative plasmids. Plasmids were transferred to S. lividans 1326 and assayed for associated cholesterol oxidase activity. Symbols: \blacksquare , choP region; \Box , chromosomal fragment from Streptomyces sp. SA-COO; —, pIJ702. Arrows show the lengths and directions of the choP and choA genes.

8.0)-5 mM EDTA-6 mg of lysozyme per ml and lysed by incubation at 37°C for 1 min. Sodium dodecyl sulfate was added to give a final concentration of 0.7%, and the suspension was heated at 100°C for 1 min. The lysate was extracted two times with phenol at 70°C. The nucleic acid was precipitated (2.5 volumes of ethanol and 0.1 volume of 3 M sodium acetate; -20°C), suspended in 2.5 M lithium chloride-20 mM EDTA (pH 7.5), and incubated for 1 h at 0°C. After centrifugation (12,000 \times g for 15 min), the pellet was washed with 2.5 M lithium chloride and suspended in 20 mM EDTA (pH 7.5). Then 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol were added, and the mixture was stored at -70° C. Samples of the suspension of RNA were centrifuged in a microcentrifuge, washed with 70% ethanol, and dried in vacuo. The final pellet was suspended in either 40 mM 3-(N-morpholino)propanesulfonic acid (MOPS; pH 7.0)-10 mM sodium acetate-1 mM EDTA (for Northern [RNA] hybridization) or 5 mM Tris hydrochloride (pH 8.2)-50 mM KCl-5 mM MgCl₂-5 mM dithiothreitol (for mappings of S1 and primer extension).

Manipulations of DNA. Preparation of plasmid DNA and transformation of S. *lividans* were performed as described previously (16).

DNA sequence analysis. Sequencing reactions were performed by the M13 dideoxy-chain termination method (32). To eliminate the G-C band compression, we used deoxy-7-deazaguanosine triphosphate instead of dGTP (18, 23). The labeled fragments were separated by polyacrylamide gel electrophoresis under denaturing conditions. The gels were fixed, dried, and exposed overnight to X-ray film.

Synthesis and purification of oligonucleotides. All oligonucleotides for primer extension mapping were synthesized with a DNA synthesizer from Biosearch, San Rafael, Calif. The oligonucleotides were subsequently purified by highperformance liquid chromatography (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with a reverse-phase column (Finepac SIL-C18).

Mappings of S1 and primer extension. For S1 mapping, RNA (20 μ g) was hybridized overnight to a 5'-end-labeled DNA fragment (>10⁶ cpm), which was present in excess (21). The hybridization temperature was 3 to 4°C above the melting temperature of the probe (63°C). The hybridization reaction was terminated by adding one-fifth of the reaction mixture to 0.1 ml of S1 buffer (0.28 M NaCl, 0.05 M sodium acetate [pH 4.6], 4.5 mM ZnSO₄) containing 300 U of S1 nuclease and incubated at 37°C for 30 min. After precipitation with ethanol, the reaction mixtures were dissolved in a sequencing dye that contained 80% formamide and run on a 6% acrylamide–8 M urea sequencing gel with labeled DNA markers.

For primer extension experiments, oligonucleotides complementary to the mRNA were mixed with total RNA (20 µg) in a total volume of 32 μ l, and 8 μ l of 5× E buffer (250 mM KCl, 25 mM Tris hydrochloride [pH 8.0], 50 mM MgCl₂, 5 mM dithiothreitol) was added. The samples were heated at 65°C for 20 min and then cooled slowly to 37°C, and the following solutions were added: 1 μ l of [α -³²P]dCTP (10 μ Ci), 5 μ l of deoxynucleoside triphosphate mixture (5 mM each GTP, ATP, CTP, and TTP), and 1 µl of reverse transcriptase (20 U). After incubation at 42°C for 1 .5 h, the samples were hydrolyzed by addition of 12.5 µl of 0.5 M NaOH, kept at 95°C for 5 min, and then chilled on ice. Subsequently, the NaOH was neutralized by adding 12.5 µl of 0.5 M HCl. The reaction mixture was precipitated with ethanol, dried, and dissolved in the sequencing dye. Each sample was loaded on an 8% acrylamide-8 M urea gel in a lane next to the corresponding sequence to determine the nucleotide at which transcription began.



FIG. 2. Production of cholesterol oxidase in the producing strain of *Streptomyces* sp. and in *S. lividans* 1326 that carried various plasmids. Symbols: \bigcirc , strain SA-COO; \bigcirc , *S. lividans* 1326(pCO-3); \triangle , *S. lividans* 1326(pCO-6); \square , *S. lividans* 1326(pCO-51), \blacksquare , *S. lividans* 1326(pCO-7).

Northern blot hybridization. Total RNA (20 μ g per lane) was fractionated on 1.5% agarose-2.2 M formaldehyde gels as described in Maniatis et al. (21). For molecular weight standards, 18S and 28S rRNAs from *Saccharomyces cerevisiae* were used. The RNA was immobilized on a Biodyne nylon membrane (Pall Bio-Support Co., East Hill, N.Y.) by the capillary method of Southern (35); the hybridization conditions were those recommended by the manufacturer of the membrane. DNA probes were labeled with [α -³²P]dCTP and the random-primer DNA labeling system of Nippon Gene Co., Toyama, Japan.

Estimation of cytochrome P-450. Late-logarithmic-phase Streptomyces cells were harvested by centrifugation at 6,000 \times g for 10 min. The wet cells were ground with a 2 \times volume of quartz sand and suspended with 0.2 M potassium phosphate buffer (pH 7.2). The homogenate was centrifuged at $18,000 \times g$ for 15 min, the supernatant was collected, and an equal volume of buffer solution (0.2 M potassium phosphate buffer [pH 7.2], 40% [vol/vol] glycerol, 0.4% [vol/vol] Emulgen 913 [Kao-Atlas Co., Tokyo, Japan]) was added. Cytochrome P-450 levels were measured as described by Omura and Sato (28) by analysis of the CO difference spectrum of dithionite-reduced samples, using 91 mM^{-1} cm^{-1} as the extinction coefficient between 450 and 490 nm. After the base line was recorded, CO was carefully bubbled through the sample cuvette for about 20 s, and the cuvettes were sealed with Parafilm. Reduction of the sample with dithionite was effected by addition of a few milligrams of

solid $Na_2S_2O_4,$ and the difference spectrum was then recorded.

Computer analysis. The nucleotide and amino acid sequences were analyzed with GENETYX programs (SDC Software Development Co. Ltd., Tokyo, Japan) and IDEAS (Integrated Database and Extended Analysis System for Nucleic Acids and Proteins) programs for the Laboratory of Mathematical Biology and the Advanced Scientific Computing Laboratory, National Cancer Institute, Frederick Cancer Research Facility, Frederick, Md., and for the Institute for Chemical Research, Kyoto University, Kyoto, Japan.

RESULTS

Promoter region for the *choA* gene. We tried to find promoter regions for *choA* by deletion analysis of pCO-3, which carries the *choA* gene (Fig. 1). Transcription and translation of pCO-6, a plasmid that lacks the 1.5-kilobase (kb) *KpnI-SacI* fragment in pCO-3, resulted in cholesterol oxidase activity (Fig. 2). pCO-3 was further truncated by digestion with *PstI*. The resultant plasmid, pCO-51, lacked the 2.1-kb *PstI* fragment and had no associated cholesterol oxidase activity. However, pCO-7, a plasmid that lacks the 0.3-kb *PstI* fragment in pCO-6, still had enzymatic activity. These results suggest that the promoter region for the *choA* gene should be found within the 0.7-kb *PstI-KpnI* fragment.

Next, the region upstream from the *choA* gene was sequenced. We found a potential promoter sequence located

GGTACCCCCGGTAACCCAACCCCCTG ¥ 100 27 CCCGTACCCGCAGGTAGCCCGCCGCGGGGGAACGATCTCG<u>TTCACAGCCTTCACATCGCCTCCATACCGTCAT</u> TOGACOG Het-Thr-Gln-Ala-Ala-Pro-Val-Thr-Phe-Ser-Thr + 174 GTC. CGT. GAG AAT. TAC. TTC. GGT. CCG. CCG. GCG. GAG. ATG. CAG. GCA. CTG. CGG. CAC. AAG. GCA. CCC Val-Arg-Glu-Asn-Tyr-Phe-Gly-Pro-Pro-Ala-Glu-Met-Gln-Ala-Leu-Arg-His-Lys-Ala-Pro GTG.ACG.CGC.ACG.GCC.TTC.GCC.GAC.GGC.CCC.GCC.GCC.TGG.CTC.ACC.GGA.TAT.TCC.GCC Val-Thr-Arg-Thr-Ala-Phe-Ala-Asp-Gly-Arg-Pro-Gly-Trp-Leu-Val-Thr-Gly-Tyr-Fer-Ala GCG.CGT.GCG.GTG.TTA.TCC.GAT.TCC.CGT.TTC.ACC.GCC.CGC.GGA.GAA.CGC.GAG.CAC.CCC.GCC Ala-Arg-Ala-Val-Leu-Ser-Asp-Ser-Arg-Phe-Thr-Ala-Arg-Gly-Glu-Arg-Glu-His-Pro-Ala 354 TG.CCG.CCG.CCC.GCC.ACC.CTG.GAG.GAC.GAG.CGC.TGC.CGC.AGG.CTG.ATC.GCC.GGA.CAG.TTC Val-Pro-Arg-Ala-Ala-Thr-Leu-Glu-Asp-Glu-Arg-Cys-Arg-Leu-Ila-Ala-Gly-Gln-Phe 414 ACC.GCC.CGC.CGG.ATG.CGG.CAG.CTC.ACC.GGG.CGG.ACC.GAG.CGG.ATC.GTC.CGC.GAA.CAC.CTC Thr-Ala-Arg-Arg-Met-Arg-Gln-Leu-Thr-Gly-Arg-Thr-Glu-Arg-Ile-Val-Arg-Glu-His-Leu 474 GAC.GCC.ATG.GAG.CAC.ATG.GGC.TCC.CCG.GCC.GAC.CTC.GAG.CAC.TTC.GCC.CTC.CCC.GTC Asp-Ale-Met-Glu-His-Met-Gly-Ser-Pro-Ale-Asp-Leu-Val-Glu-His-Phe-Ale-Leu-Pro-Val * 534 CCC.TCG.CTC.GTC.ATC.GCC.GAA.CTG.CTC.GGC.GTG.CCC.CCC.GCC.GAC.CGG.GAG.CAG.TTC.CAG Pro-Ser-Lew-Val-Ile-Ala-Glu-Lew-Gly-Val-Pro-Pro-Ala-Asp-Arg-Glu-Gln-Phe-Gln CAC.GAC.ACC.CTC.CGG.TGG.GGC.GGC.TTC.GGC.CGG.TCC.ACC.GAG.GAG.GTC.ACC.GAG.GCC.TTC His-Asp-Thr-Leu-Arg-Trp-Gly-Gly-Phe-Gly-Arg-Ser-Thr-Glu-Glu-Val-Thr-Glu-Ala-Phe Pstl 654 GTC. TC. GTC. GGC.GGC.CAA.<u>CTG.CAG</u>.CGC.CTC.GTG.CGG.CTC.AAG.CGC.ACC.GAG.CCG.GGC.GAC Vel-Ser-Leu-Gly-Gly-Gln-Leu-Gln-Arg-Leu-Vel-Arg-Leu-Lys-Arg-Thr-Glu-Pro-Gly-Asp GAC.CTC.CTC.AGC.GGC.CTG.ATC.GCC.GCC.GAC.CCC.GCG.CTC.ACC.GAC.GAG.GAA.CTC.GCC.TCG Asp-Leu-Leu-Ser-Gly-Leu-Ile-Ala-Ala-Asp-Pro-Ala-Leu-Thr-Asp-Glu-Glu-Leu-Ala-Ser 774 ATC.GCC.TTC.CTG.TTG.CTG.GTC.GCC.GGC.CAC.GGG.ACC.ACC.GCC.CAC.CAG.ATC.GCC.CTC.GGC 11e-Ala-Phe-Leu-Leu-Leu-Val-Ala-Gly-His-Gly-Thr-Thr-Ala-His-Gln-Ile-Ala-Leu-Gly 834 GCC. TTC. CTG. CTG. CTC. GAA. CAC. CCC. GAC. CAG. CTG. GCC. GCG. CTG. CGC. GCC. GAC. CCC. GCC. CTG Ala-Phe-Leu-Leu-Clu-His-Pro-Asp-Gln-Leu-Ala-Ala-Leu-Arg-Ala-Asp-Pro-Ala-Leu ACG.GAG.AGC.GCC.GCC.GAG.GAG.CTG.CTG.CGC.CAC.CTG.AGC.GTC.GTC.CAC.CAC.GGC.CCC.ACC Thr-Glu-Ser-Ala-Val-Glu-Glu-Leu-Leu-Arg-His-Leu-Ser-Val-Val-His-His-Gly-Pro-Thr Psti + 954 GT.GCC.GCC.CTG.CAG.GAC.GCC.GAC.ATC.GAG.GGC.ACC.CCC.GTC.AAG.GCG.GGC.GAG.GTC.GTC Arg-Ala-Ala-Lau-Gin-Asp-Ala-Asp-Ile-Glu-Gly-Thr-Pro-Val-Lys-Ala-Gly-Glu-Val-Val + 1014 GTG.GTC.TCG.CTC.GGC.GCC.GCC.AAC.CGC.GCC.CCC.GCA.CGC.TTC.GAG.CGG.CCC.GAC.GCC.GTG Val-Val-Ser-Lau-Gly-Ala-Ala-Asn-Arg-Asp-Pro-Ala-Arg-Phe-Glu-Arg-Pro-Asp-Ala-Val + 1074 GAT.GTC.ACC.CGC.GAG.GAC.ACC.GGC.CAC.CTC.GCC TTC.GGG.CAC.GGC.ATG.CAC.CAG.TGC.CTC Asp-Val-Thr-Arg-Glu-Asp-Thr-Gly-His-Leu-Ala Phe-Gly-His-Gly-Het-His-Gln-Cys-Leu * 11j4 GGC.CGG.CAA.CTG.GCC.CGC.ATC.GAA.CTG.CGC.GTC.GCC Gly-Arg-Gln-Leu-Ala-Arg-Ile-Glu-Leu-Arg-Val-Ala Leu-Thr-Ala-Leu-Glu-Arg-Phe CCG.CAC.CTG.CGC.CTG.GCC.TGC.CCC.GCG.GCG.GAG.ATC.CCG.CTC.CGC.CAC.GAC.ATG.CAG.GTC Pro-His-Leu-Arg-Leu-Ala-Cys-Pro-Ala-Ala-Glu-Ile-Pro-Leu-Arg-His-Amp-Met-Glm-Val TAC.GGC.GAC.CGG.CTG.CCG.GTC.GCC.TGG.TGA CCGTCCCCGTCACCCCCCGAACCACCTTCCCCAC Tyr-Gly-Ala-Asp-Arg-Leu-Pro-Val-Ala-Trp-*** 1384 + 1322 ChoA ----> 1384 CCCTGAAGAAACGTCTCG<u>GAAAGG</u>TCAGGTAGACCCCCC GTG.ACT.GCA.CAA.CAG.CAC.CTG.TCC.CGC.CGC 8 D Met-Thr-Ala-Gln-His-Leu-Sør-Arg-Arg

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FIG. 3. Nucleotide sequence of the 1.4-kb fragment upstream from the *choA* gene and the predicted amino acid sequence of *choP* (GenBank/EMBL accession no. is M31939). An enclosed rectangle shows the sequence that is highly homologous to sequences of other cytochrome P-450 genes. The presumptive ribosome-binding site (SD; underlined), possible promoter regions (-10 and -35), and predicted sites of initiation of transcription (\P) are indicated. The DNA sequence complementary to the synthetic oligonucleotides used for primer extension mapping are represented in italics with dots.



FIG. 4. Low-resolution S1 nuclease mapping of the *choP* and *choA* regions. RNAs were isolated from S. *lividans* 1326(pCO-6) and hybridized to the 5'-labeled probe of a 667-bp *PstI-KpnI* fragment in the *chop* region (A) or to a 644-bp *Sau3AI* fragment in the *choA* region (B). Products treated with S1 nuclease were analyzed by gel electrophoresis on 6% acrylamide-8 M urea gels. Lanes: 1, sample without S1 nuclease; 2, sample with S1 nuclease. Arrowheads indicate bands described in the text. The marker positions refer to a *HaeIII* digest of pUC119.



FIG. 5. Primer extension mapping of the *choP* (A) and *choA* (B) regions. The primer extension method is described in the text. Primer extension products were analyzed by electrophoresis on 8% acrylamide-8 M urea gels. The corresponding sequences of the *cho* genes are shown on the left. Arrowheads indicate the probable in vivo transcription initiation sites for the promoters. Lanes: 1, RNA prepared from S. lividans 1326(pIJ702); 2, RNA prepared from S. lividans 1326(pCO-6).



FIG. 6. Northern blot analysis of transcripts from the *choP* and *choA* regions. (A) Autoradiograms of Northern blots of transcripts; (B) diagrams of DNA probes and possible locations and sizes of transcripts. Lanes: 1, *S. lividans* 1326(pCO-3); 2, *S. lividans* 1326(pCO-6). The 18S (1.9-kb) and 28S (4.7-kb) rRNAs from yeast cells, used as size markers, were fixed with 1 M acetate and stained with 0.2% methylene blue.

about 1.3 kb upstream from the *choA* gene. Unexpectedly, we also found an ORF capable of coding for a 41-kilodalton protein. This ORF was designated *choP*.

Nucleotide sequence of *choP*. The new ORF contains 1,039 base pairs (bp), with an ATG initiation codon at position 141 and a TGA termination codon at position 1284 (Fig. 3). The initiation codon is preceded by a sequence with a high degree of similarity to the -10 and -35 consensus sequence (TTGaca-18 bp-tAGgaT) and has a potential ribosomebinding site (GAAAGG), showing complementarity to the 3' end of *Streptomyces* 16S rRNA (4). At a site 74 bp downstream from the TGA termination codon, we located the structural gene for *choA*. A potential ribosome-binding site (GAAAGG) was also found in this spacer region. However, no long palindromic sequence, serving as a transcription terminator (31), was found between the *choP* and *choA* genes.

Initiation of transcription of choP and choA. The 5' termini of transcripts of choP and choA generated in vivo were determined by S1 nuclease mapping and primer extension with reverse transcriptase. Cellular RNAs were isolated from S. lividans 1326 that carried pCO-6. The RNAs were hybridized with a probe, either a 667-bp PstI-KpnI fragment in the choP gene (probe 1) or a 644-bp Sau3AI fragment in the choA gene (probe 2), treated with S1 nuclease, and then analyzed by polyacrylamide-urea gel electrophoresis with markers for size determination (Fig. 4). When probe 1 was hybridized with the RNAs, we observed a protected fragment of 570 bases. Probe 2 gave a protected fragment of 490 bases.

To determine more precisely the 5'-terminal sites of tran-

scripts, we analyzed the transcripts by primer extension mapping. Oligonucleotides (18-mers [CGGCGGACCGAAG TAAT and GAAGGCGGCCATGCCGAG] complementary to sequences in *choP* and *choA*, respectively) were synthesized as primers (Fig. 3). The radioactively labeled synthetic primers were annealed with the respective complementary sequences and then extended with reverse transcriptase. Transcription of the *choP* gene was found to start at the C residue that is 41 bases upstream from the ATG initiation codon (Fig. 5). We also found that the synthesis of mRNA starts at the C residue at position 1384, which is located within the structural gene for *choA* (Fig. 3). The two mRNA start sites agree with the sizes of protection fragments obtained from the S1 mapping analysis.

Northern blot analysis. The sizes of transcripts of the choP and choA genes in vivo were determined by Northern blot analysis. Total RNA was prepared from S. lividans 1326 cells that carried pCO-3 or pCO-6. A 0.29-kb DNA fragment of the choP gene and a 0.33-kb DNA fragment of the choA gene were used as intergenic probes (Fig. 6). Results from autoradiography revealed that at least three species of RNA were transcribed. Transcripts of 2.9, 1.8, and 1.6 kb were observed; the 2.9-kb transcript hybridized to the two probes and was identical in size to the theoretical transcript from the site of initiation of transcription of mRNA for choP to a predicted termination site for choA, which is located 5 bp downstream from the TAA termination codon of choA (16). The 1.8- and 1.6-kb transcripts bound to probes 1 and 2, respectively, but not vice versa. These 1.8- and 1.6-kb transcripts may start from the initiation site in the choP and choA genes, respectively, although their functions are unknown.

These results of S1 and primer extension mappings and Northern (RNA) analysis suggest that the *choP* and *choA* genes are transcribed polycistronically from the promoter upstream from the *choP* gene to include transcription of the *cho* operon.

Utilization of codons in the *choP* gene and G+C content. Codon usage in the *Streptomyces choP* gene was analyzed. The overall G+C content of the *choP* gene is 73.1%, which is reflected in the third position of the codons used (92.1%), well within the range found in previously sequenced *Streptomyces* genes (8, 9, 12, 14, 16, 30).

Amino acid sequence of choP. The amino acid sequence deduced from the ORF (choP) indicates that the product of choP contains 381 amino acid residues, having a molecular weight of 41,668. We have searched for homologies in the peptide sequences of the choP gene and other genes. We found that the sequence is partially homologous to sequences of cytochrome P-450 genes from several organisms, such as those for P-450cam from Pseudomonas putida (3) and human P-450c21, (steroid 21-hydroxylase; 26) (Fig. 7). In particular, the sequence from amino acids 332 to 352 (Fig. 3) has a high degree of sequence homology to Pseudomonas P-450cam (P450CIA1; 3), yeast lan (P450LIA1; 26), fish P_1 -450 (P450IAI; 26), chicken P-450IIc (P450IIC10; 11), bovine P-450(SCC) (P450XXIIA1; 24), porcine c17 (P450XVIIA1; 26), rabbit form 2 (P450IIB1; 26), rat P450 pcn 1 (P450IIIA1; 10), mouse P₃-450 (P450IA2; 20), and human P450c21 (P450XXIA; 38) (Fig. 8). These homologous regions, each containing a cysteine residue, are known as the sites of binding heme iron in cytochrome P-450s (26).

Estimation of cytochrome P-450. We examined the product of the *choP* gene from the parent strain *Streptomyces* sp. strain SA-COO and from *S. lividans* 1326 with or without pCO-3. The cell extracts prepared from strain SA-COO and

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250 Gldtvvnfi Gegttabq	25 LSPSMEP 230 I Algapl	O LAKSP I I	EURQ D-QLA-	ELIQRI Malradi	PERIPAN	CEELLRR	PSLVADG IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	30 RIL # -PTRAA	O TSDYE ¥28 LQDAD	30(P∎GVQI 0 ∰ 1 BGTP\) LEEGDQ IIII VEAGEV	I LL PQN VVVSLG
250 Gldtvvnfi S BGttabq	25 LSFSMEF 230 I Algapl 2	O LAKSP # # Llehp	EURQ D-qla-	ELIQRI Malradi	PERIPAA ALTESA	CEELLRR NAMES VEELLRE	FSLVADG IIIIII LSVVIIIG	30 Ril # -Ptraa # #	O TSDYE ¥28 LQDAD	30(PEGVQI 0 # 1 BGTP1 # #) I K K GDQ II II V K A GB V II II II II	ILL PQN VVVSLG
250 GLDTVVNFI E GUGTTABQ E I QQRLQE	25 LSFSMEF 230 I Algapl # # -EldMel	O LAKSP LLENP GPGAS	EHRQ D-QLA- SSRVPY	ELIQRI MALRADI KDRARI	PERIPAA Altesa Pllna1	CEELLRE	FSLVADG IIIII LSVVIIIG III RPVVPLA	30 RIL # -PTRAA # # LPHRTT	O TSDYE ¥28 LQDAD	30(PEGVQI 0 # IBGTPV # # ISGYDI) LEEGDQ III VEAGEV IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	ILLPQN VVVSLG IIPNLQ
250 GLDTVVNFI ¥ ¥ GHGTTABQ EIQQRLQE	25 LSPSMEP 230 I Algapl # # -Eldmel	O LAKSP ILLENP GPGAS	EHRQ D-QLA- SSRVPY	ELIQRI MALRADI MARARI	PERIPAA ALTESA PALTESA PLLNA1	CEBLLRR	FSLVADG IIIII LSVVBBG III RPVVPLA	30 RIL # -PTRAA # # LPHRTT	O TSDYE ¥28 LQDAD -RPSS	30(PEGVQI 0 # I EGTPV # # I SGYDI	CENTRACES	ILLPQN VVVSLG I IPNLQ
250 GLDTVVNPI XX GHGTTARQ EIQQRLQE	25 LSPSMEF 230 I Algapl # # -Eldmel	O LAKSP ILLEHP GPGAS	ENRQ D-QLA- SSRVPY	ELIQRI ALRADI KDRARI 31	PERIPAA ALTESA PALTESA PLLNA1 30	CEELLRR MANNA VEELLRE I IAEVLRL ISO	FSLVADG IIIII LSVVBBG III RPVVPLA	30 RIL # -PTRAA # # LPHRTT	IO .TSDYE ¥28 LQDAD :-RPSS	30(PEGYQI 0 # 1egtpy # # 1sgydi) # # VEAGEV # # IPEGTV	ILL PQN VVVSLG I IPNLQ
250 GLDTVNPI E E GHGTTAHQ E I QQRLQE LSGLDEREI	25 LSPSMEF 230 I Algapl # # -Eld H EL NAC PME -	O LAKSP LLBHP GPGAS	EURQ D-QLA- SSRVPY FSRQKY	EL I QRI MALRADI KDRARI SH-TTI	PERIPAA ALTESA PALTESA PLLNA1 50 Gugsel	CEELLRE MANNEN VEELLRE NAEVLRL SSO CLGQULA	FSLVADG IIIII LSVVDHG III RPVVPLA RRBIIVT	30 RIL # -PTRAA # # LPHRTT LEBVLT	O TSDYE ¥28 LQDAD -RPSS RIPDP	30(PEGVQI 0 # 1BGTP\ # # 1SGYD1 SIA-P() I I I I VE AGE V II II I PEGT V GAQIAII	ILL PQN VVVSLG IIPNLQ KSGI
250 GLDTVVNPI GNGTTABQ EIQQRLQE- LSGLDEREI 300 %	25 LSPSMEF 230 I ALGAPL # ELDBEL NACPME-	O LAKSP ILLBHP GPGAS -VD	EURQ D-QLA- SSRVPY PSRQKY # #	ELIQRI MALRADI KDRARI SH-TTI M	PERIPAA ALTESA PLLMAT Gugsbl	CEELLRE MEELLR	FSLVADG LSVVNNG RPVVPLA RRBIIVT	30 RIL # PTRAA # # LPHRTT LKEVLT # #	O TSDYE #28 LQDAD -RPSS RIPDP # #	30(PEGVQI 0 # 1EGTP\ # # 1SGYD SIA-P(# #) I K KGDQ I II V KAGBY I KAGBY I PEGTY GAQIAN II I II	VILL PQN VVVSLG II VIPNLQ KSGI 370
250 GLDTVVNPI GIGTTABQ EIQQRLQE LSGLDERRI 300 AANRDPARI	2 S LSPSMEF 2 30 I ALGAPL # -ELDHEL NACPMH- # PERPDA-	0 LAKSP LLEHP GPGAS -VD ** -VD	EHRQ D-QLA- SSRVPY PSRQKY \$ \$ TREDT	ELIQRI MLPADI KDRARI SH-TTI M GH-LAI	PERIPAA ALTESA PLLNA1 GIGSBL	CEELLRE MANNES VEELLRE MAEVLRL SO CLGQULA MANNES CLGRQLA	FSLVADG LSVVING RPVPLA RRBIIVT	30 RIL # -PTRAA # # LPHRTT LKEVLT # # LTALLE	IO TSDYE #28 LQDAD -RPSS RIPDP # # RPPHL	30(PHGVQI 0 # IBGTPV # # ISGYDI SIA-P(# # RLACP/) LEKGDQ IEKGBY IPEGTY GAQIAN IIII IIIII IIIII IIIII IIIIII IIIIII	ILLPQN VVVSLG IIPNLQ IIPNLQ KSGI 370 LRHDNQ
250 GLDTVANFI E E GIIGTTAIQ E I QQRLQE LSGLDRREI 300 AANEDPARI E T	2 S LSPSMEP 2 30 I ALGAPL # - ELDHEL NACPMH- # FERPDA- 1 3 T T	0 LAKSP # # LLBHP GPGAS 	EHRQ D-QLA- SSRVPY PSRQKY \$ \$ TREDT	ELIQRI ALRADI KDRARI SH-TTI GH-LAI	PERIPAA ALTESA PLLNA1 SO GRGSBL	CEELLRE VEELLRE VEELLRE So CLEQUILA SCI QUILA CLEQUILA CLEQUILA	PSLVADG XXX LSVVHG XXX RPVVPLA RRBIIVT XXX XXXX XXXX XXXXX XXXXXXXXXXXX	30 RIL # -PTRAA # # LPBRTT LKBVLT # # LTALLE # #	IO TSDYE 28 LQDAD -RPSS RIPDP R RPPEL 8	30(PHGVQI 0 # IBGTP\ # # ISGYDI SIA-P(# # RLACP/) LEKGDQ VEAGEV VEAGEV VEAGEV SAQIAN XEI-P XEI-P	ILLPQW VVVSLG IPNLQ IPNLQ KSGI 370 LRHDNQ I RHDNQ
250 GLDTVANPI E E GHGTTABQ E I QQRLQE LSGLDERRI 300 AANRDPARI GABLDRTVI	2 S LSPSMEP 2 30 I ALGAPL # - ELDHEL NACPMH- # PERPDA- 1 TT VERPMEP	O LAKSP LLEHP GPGAS -VD ¥¥ -VD ¥ ¥ VPDRP	EHRQ D-QLA- SSRVPY FSRQKY TREDT LEPGKN	ELIQRI ALRADI KDRARI SH-TTI GH-LAI SRALAI	PERIPAA ALTESA PLLNA1 SO GRGSBL GRGSBL	ICEELLRE MANNEN VEELLRE I MANNEN VEELLRE I MANNEN SO CLEGRULA ICLEGRULA ICLEGRULA	PSLVADG S S S LSVVHG RPVVPLA RRBIIVT S S RIBLRVA RIBLRVA RLBLPVV	30 RIL # -PTRAA # # LPBRTT LKBVLT # Etalle # Etalle # Etalle	O TSDYE #28 LQDAD -RPSS RIPDP # # RPPHL # # APTLL	30(PEGVQI 0 # IBGTPV # # ISGYDI SIA-P(# # ELACP/ PSGDAI) LEKGDQ # # VEAGEV # # IPEGTV GAQIAH # # AAEI-P # LPSLQP	ILLPQM VVVSLG IPNLQ IPNLQ STO LRUDWQ I RUDWQ I RUDWQ
250 GLDTVANPI E GAGTTABQ E I QQRLQE LSGLDERRI 300 AANEDPARI E GABLDETVI	25 LSFSMEP 230 IALGAPL -ELDHEL WACPME- FREPDA- STT VERPMEP (00	O LAKSP LLEHP GPGAS -VD VD VD VD VPDRP	EURQ D-QLA- SSRVPY PSRQKY \$ \$ TREDT LEPGKN	ELIQRI ALRADI KDRARI SH-TTH GH-LAF SRALAF	PERIPAA PALTESA PLLNA1 SO GIGSSIL	ICEELLRE VEELLRE VEELLRE VIAEVLRL SO CLGQQUAA VIAEVLRL SO CLGQQUAA VIAEVLRL SO CLGQQUAA	FSLVADG LSVVHG RPVVPLA RRBIIVT RIBLRVA RIBLRVA RLBLPVV	30 RIL # -PTRAA # # LPHRTT LTRLLU # # LTALLU # # LTRLLU	O TSDYE #28 LQDAD -RPSS RIPDF # # RPPHL # # APTLL 450	30(PEGVQI 0 # IBGTPV # # ISGYDI SIA-P(# # RLACP/ PSGDAI) LEKGDQ WAGBV WAGBV MAGEV AQIAN MAGI-P LPSLQP	ILLPQM VVVSLG I IPNLQ ISGI 370 LRIDMQ I RIDMQ I PH
250 GLDTVVNPI GIGTTABQ EIQQRLQE LSGLDERRI 300% AANRDPARI 3 GANLDETVI 400	25 LSFSMEP 230 IALGAPL -ELDHEL WACPMU- FREPDA- STEP VERPMEP (00	0 LAKSP LLBHP GPGAS -VD VD YD YPDRP	EURQ D-QLA- SSRVPY PSRQKY \$ \$ TREDT Lepgkn	ELIQRI ALRADI KDRARI SH-TTH GN-LAF SRALAF	PERIPAA ALTESA PLLNAT GIGSHL GIGGHG GCGARN	CCEBLLER NUTER I INTER I INTER	PSLVADG S S S LSVVHA RVVPLA RRBIIVT S S RIBLRVA S LELPVY	30 RIL # -PTRAA # # LPHRTT LTRLLU # # LTALLU # Etrllu	O TSDYE #28 LQDAD -RPSS RIPDP R RFPHL R AFTLL 450	30(PHGVQI 0 # IBGTP\ # # ISGYD SIA-P(# # RLACP/ PSGDAI) LKKGDQ # # IPEGTV GAQIAM # # AAEI-P # LPSLQP	ILLPQN VVVSLG IPNLQ IPNLQ KSGI S70 LRNDNQ IRNDNQ IPN
250 GLDTVANFI GIGTTABQ EIQQRLQE- LSGLDEREI 300% AAREDPARI S GANLDETVI 400 VSGVQALPI	25 L.SPSMEF 230 I ALGAPL -ELDMEL MACPMH- FERPDA- 217 YERPMEP 400 L VW	0 LAKSP # LLBHP GPGAS - VD ¥ ¥ VD ¥ ¥ VPDRP	EURQ D-QLA- SSRVPY PSRQKY # # TREDT LEPGKN	ELIQE ALRADE KDRAEI SH-TTF GH-LAF SRALAF	PERIPAA ALTESA PLLNAT Gugshi Gugshi Gugghi Gugghi Gugghi	CCEELLRE MANNA VEELLRU I MANNA I MANNNA I MAN	FSLVADG SSLVADG LSVVHG RPVVPLA RRBIIVT SSL RIBLRVA SLELPVV	30 RIL # -PTRAA # # LPHRTT LKBWLT LKBWLT # LTALLE # # LTALLE # LTRLLQ	O TSDYE #28 LQDAD -RPSS RIPDP R RPPHL R AFTLL 450	30(PHGVQI 0 # IBGTP\ # # ISGYD SIA-P(# # RLACP/ PSGDAI) LKKGDQ # # VKAGBV # # IPEGTV GAQIAM # # AAEI-P # LPSLQP	ILLPQM VVVSLG IPNLQ STO LRIDMQ I PH
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250 GLDTVANFI E GIGTTARQ E I QQRLQE- LSGLDRRRI 300 AARRDPARI E T GANLDETVI 400 VSGVQALPI Y YGADRLPY	25 LSPSMEP 230 I ALGAPL # # - ELDHEL MACPME- # PREPDA- ETT VERPMEP 400 L VW : : VAW	0 LAKSP LLEHP GPGAS -VD ¥ VD ¥ VPDRF	EURQ D-QLA- SSRYPY PSRQKY \$ \$ TREDT LEPGKN	ELIQRI MALRADI KDRARI SH-TTH GH-LAF SRALAF	PERIPAA ALTESA PLLNAT Gugshi Gugshi Gughto Gugary	CEBLLER WELLER SO CLGQULA CLGQULA	PSLVADG SVUNG RPVVPLA RRBIIVT RIBLRVA RIBLRVA RIBLRVA	30 RIL # # LPHRTT LKBWLT # Etalle # Etalle # Etalle	O TSDYE #28 LQDAD - RPSS RIPDP # FIL RFPE AFTLL 450	30(PHGYQI 0 # IBGTPI # # ISGYDI SIA-P(# # RLACP/ PSGDAI) LEEGDQ # # IPEGTY GAQIAM # # NAEI-P LPSLQP	VILLPQM VVVSLG IIPHLQ IIPHLQ ISGI 370 LRIDMQ IRIDMQ IRIDMQ IPH

FIG. 7. Comparison of sequences of the *choP* gene product, cytochrome P-450cam from *P. putida*, and human cytochrome P-450c21 (steroid 21-hydroxylase). Numbers refer to amino acid positions from the NH_2 terminus of each protein. Identical amino acids are indicated by shaded boxes.

from strain 1326 that carried pCO-3 exhibited a CO difference spectrum typical of the CO complex of the reduced form of cytochrome P-450, with an absorption maximum at 450 nm, whereas this peak was very small in the extract from

		-7	0	7	13
Streptomyces choP			ME Q CER E	Q	LR業A
Pseudomonas P-450	CAM (CI)		SHL CARE Q	H Mar ii	II¥T
Yeast lan	(LI)	KR G (RËRËI	HF#YCQ	LG¥L
Fish P1-450	(1))KËRËI 🗰	A I G 🗱 🗱	VELE
Chicken P45011C	(11)	₿ SA)	Ë K 🖁 I 诺A 🎆	G Man ik	
Bovine P450sec	(XIA)		ŧv #Q #v #R	R I ŽELŽ	NT 🎆
Porcine c17	(XVII)		PËSËV 🗰	M Mari q İ	L
Rabbit form 2	(11)	₿SL	K 🛛 I 🗰	G I 🗱 T 🕷	L
Rat P450sec	(111)		ËPËNË I ËM	RPËLMN	MK 🗒 A
Mouse P3-450	(1)		K N R S I 🗰	I PÄKVÄ	V
lluman P450c21	(XXI)				LEYV

FIG. 8. Comparison of the amino acid sequence of the highly conserved cysteine-containing region of the product of the choP gene with sequences of the heme-binding regions of cytochrome P-450s from various organisms. Numbers represent amino acid positions relative to the cysteine that binds the heme iron. References are given in the text. Notations in parentheses are the recommended designations for the P-450 gene family (27). Identical amino acids are shaded.

S. lividans without plasmid (Fig. 9). An absorption maximum at 420 nm is often seen in a CO difference spectrum that may be derived from heme proteins in crude extracts (22). However, the product of the *choP* gene seemed to be unstable in S. lividans cells, in view of the rather broad difference spectrum obtained with the extract from strain 1326 when it carried pCO-3.

DISCUSSION

This report describes the localization and determination of the nucleotide sequence of the promoter region for the choA gene, the sizes of transcripts in the cho region, and the identification of a cytochrome P-450-like gene in the choP gene from *Streptomyces* species.

A new ORF (*choP*), found in the *cho* gene, is located 70 bp upstream from the *choA* gene. The deletion analysis, nucleotide sequence, and data from S1 and primer extension mappings and Northern (RNA) blot analysis suggest that the *choA* gene is transcribed from the promoter region of the *choP* gene and that the *choP* and *choA* genes are transcribed polycistronically and form an operon. We have designated this operon the *cho* operon.

By low-resolution S1 mapping and primer extension mapping, we found two sites of initiation of transcription in the *cho* region. However, one site is located in the structural gene for *choA* (Fig. 3 to 5). Since the region upstream from



FIG. 9. CO difference spectra of cell extracts of *Streptomyces* sp. strain SA-COO and of S. lividans 1326 with or without plasmid. The analytical method for generating the CO difference spectra is described in the text. Symbols: —, S. lividans 1326(pCO-3); --, Streptomyces sp. strain SA-COO; —, S. lividans 1326.

this site is essential for expression of cholesterol oxidase, transcription of mRNA from the choA gene should start from the other site of initiation of transcription, which is located 41 bases upstream from the ATG initiation codon of choP. This conclusion is supported by the results of Northern blot analysis (Fig. 6). We found a transcript of the size (2.9 kb) predicted from the location of the first initiation site and a putative termination site in a long inverted-repeat sequence of choA. However, we also found two additional transcripts, of 1.8 and 1.6 kb. Although we have not yet determined their sites of termination, the 1.8-kb transcript may be transcribed from the same initiation site as is the 2.9-kb transcript. The 1.6-kb transcript may be transcribed from the initiation site that is located 22 bases downstream from the GTG initiation codon of the choA gene. Such heterologous transcripts of one operon have been found previously in Streptomyces species (12, 37). The heterologous transcripts may be a result of a heterogeneity of RNA polymerase in Streptomyces species (6, 29). However, we could not rule out the possibility that these heterologous transcripts were products of degradation from the polycistronically transcribed mRNA.

Although we unexpectedly found the cytochrome P-450like gene in the *cho* operon, the existence of this gene in the *cho* region may not be surprising. Recently, cytochrome P-450s have been found in several *Streptomyces* species. These cytochrome P-450s are thought to be involved in the biosynthesis of antibiotics, such as the isoflavonoid genistein in *Streptomyces griseus* (33), the herbicide-inducible cytochrome P-450 in *S. griseus* (7), and the compactin-inducible cytochrome P-450sca in *Streptomyces carbophilus* (22), and in the hydroxylation of paravastatin in *Streptomyces flavovirens* SANK63684 (T. Manome et al., Abstr. Jpn. Agric. Biol. Chem. **63**:157, 1989). Furthermore, cytochrome P-450 in *Saccharopolyspora erythraea* involves 6-deoxy-erythronolide B synthesis (34). However, genes for these *Streptomyces* cytochrome P-450s have not been analyzed. Therefore, this paper may be the first report of the structure of a gene, and the analysis of its transcripts, for the cytochrome P-450 in *Streptomyces* species.

Sequence homology between the *choP* product and cytochrome P-450s is apparent from the NH_2 -terminal to the C-terminal ends. Particularly striking is the invariant sequence of 21 amino acids found near the carboxyl-terminal end of *choP* product as well as in all other cytochrome P-450s examined (Fig. 8). This region contains a cysteine residue that interacts with the heme iron moiety (26). From these preliminary comparisons of sequence homology and the detection of a typical CO difference spectrum for cy-

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tochrome P-450 from the parent strain of *Streptomyces* sp. and from *S. lividans* that carries a plasmid with the *choP* gene, it would seem reasonable to speculate that the gene product of *choP* may also adopt a similar structure in terms of a binding domain for heme iron, and it too may be involved in the metabolism of cholesterol, although we have not yet found the natural substrate for the *choP* protein or the inducer for the *cho* operon. Greater insight into the role of cytochrome P-450 in the biosynthesis of antibiotics and sterol metabolism in *Streptomyces* species must await the outcome of further genetic and biochemical studies.

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