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Minimal Requirements of the *Streptomyces lividans* 66 *oriC* Region and Its Transcriptional and Translational Activities

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Deletion analysis of a previously constructed minichromosome revealed that a stretch of DNA which is longer than 623 bp but shorter than 837 bp is required for autonomous replication of the *Streptomyces lividans* chromosome. Each of the *dnaA* and *dnaN* genes flanking the *oriC* region is individually transcribed from two promoters. Within the intergenic, nontranslatable region between the *dnaA* and *dnaN* genes, five main transcripts and several less abundant transcripts of various lengths as well as one of the promoters were identified. The introduction of additional DnaA boxes in *S. lividans* led to a significant increase in *dnaA* gene transcripts and to an enhanced level of the DnaA (73-kDa) protein. In summary, the data suggest that *dnaA* gene transcription is autoregulated and that initiation of the *S. lividans* chromosome is tightly controlled.

The replication of several bacterial chromosomes was found to be initiated at the origin of replication, *oriC*. The features of *oriC* and, with a few exceptions, the arrangement of genes in its vicinity are remarkably similar among eubacteria (25, 38). Sequence analyses revealed that the origins of various eubacteria contain highly conserved segments which are separated by variable spacer regions. The DnaA box motifs (TTATC/AC AC/AA) (36) constituting the binding sites for the initiator protein DnaA are prominent in the conserved parts of *oriC* regions from various bacteria. The only relevant variation is an A-to-G exchange at the third position of the DnaA box motif within GC-rich DNA present in gram-positive bacteria such as *Micrococcus luteus* (10) and *Streptomyces lividans* (42). Most eubacteria contain a block of genes, *dnaA-dnaN-recF-gyrB* (10, 38), encoding the initiator protein DnaA, the β-subunit of DNA polymerase III holoenzyme, a product for recombination, and the β-subunit of DNA gyrase, respectively. Within most bacteria, *oriC* is situated close to the *dnaA* gene. In *Escherichia coli*, the arrangement of the *dnaA* region matches that in other bacteria; however, this region is located about 42 kb away from *oriC* (35).

Procaryotic chromosomal replication has been best studied in *E. coli*. Its DnaA protein is a basic protein of 52 kDa to which ATP binds. About 10 to 40 ATP-DnaA molecules interact with four DnaA boxes within *oriC* (19, 36). According to a current model (14), the newly synthesized DnaA protein is titrated by these DnaA boxes. As soon as the number of DnaA molecules exceeds the number of boxes, initiation is supposed to occur. A high number of GATC sites which can be methylated by the Dam methyltransferase have been found in the *oriC* region of *E. coli*, as well as in other members of the family *Enterobacteriaceae* (44), and GANTC sites are present in the *Caulobacter crescentus oriC* (43).

Contrary to many bacteria, *Streptomyces* species grow as substrate mycelia which differentiate to aerial mycelia and spores upon depletion of nutrients. Depending on the species, the size of the GC-rich *Streptomyces* chromosome ranges between 6.6 and 8 Mb (18, 32). Recently, we identified the *oriC* region of the *S. lividans* 66 chromosome after a 2.6-kb chromosomal fragment had been cloned together with the thiostrepton resistance fragment as an autonomously replicating minichromosome (42). Sequence analysis showed that the *oriC* region of *S. lividans* 66 is identical to that of *Streptomyces coelicolor* A3(2) (7). Seventeen DnaA boxes are arranged in two clusters flanked by the *dnaA* and *dnaN* genes (42). Here we report the minimal requirements of the *S. lividans oriC* region as well as its transcriptional and translational activities.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Streptomyces* and *E. coli* strains and plasmids used and their origins are listed in Table 1.

Culture conditions and transformations. *E. coli* strains were grown in Luria-Bertani medium (27). *S. lividans* strains were cultivated on agar plates containing complete medium, as previously described, until sporulation occurred (16). Spores were used to inoculate SLAB and YEME liquid media (9, 16). Propagation and transformation of *E. coli* and *Streptomyces* strains have previously been described (16, 27). *Streptomyces* transformants were selected for resistance to 10 μg of thiostrepton per ml.

Chemicals and enzymes. Standard chemicals were obtained from Sigma and Serva. Restriction enzymes, T4 polynucleotide kinase, alkaline phosphatase, S1 nuclease, and ligase were purchased from Gibco BRL. Isotopes were supplied by Amersham.

Determination of catechol dioxygenase activity. Transformants were streaked on solid SLAB medium, and after 2 to 4 days of growth at 30°C, they were sprayed with a 0.5 M catechol solution and inspected for the yellow color of hydroxymuconic semialdehyde (8, 9). Positive colonies were grown in 50 ml of SLAB liquid medium at 30°C on a rotary shaker. Mycelia were washed in 20 ml of 20 mM potassium phosphate buffer (pH 7.2) and suspended in 1 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 10% acetone. Crude extracts were obtained after ultrasonication (Branson sonifier) and removal of cell debris by centrifugation (20,000 × *g*, 20 min). Aliquots were tested with the substrate catechol as described before (8, 9).

DNA isolation and manipulations. Total and plasmid DNAs were isolated from *Streptomyces* strains as described by Hopwood et al. (16). Single-stranded M13 phage DNA was extracted from *E. coli* by the method of Sambrook et al. (27). The methods used for purification of DNA fragments, Southern hybridization, and preparation of DNA probes have previously been described (27).

RNA isolation. *S. lividans* was grown in YEME medium containing 34% sucrose (16). After 24 to 30 h at 30°C, mycelia were harvested and the total cellular RNA was isolated by the method of Hopwood et al. (16). The RNA concentration was determined by measuring the A_{260} (A_{260} = 40 μg).

Primer extension. A synthetic oligonucleotide complementary to the first 25 nucleotides of the coding region was 5′ end labelled with γ-^{32}P and T4 polynucleotide kinase. Primer extension was performed by the method of Ausubel et al. (3) with the modifications described by Zakrzewska-Czerwińska et al. (41).

High-resolution S1 nuclease mapping. For S1 nuclease mapping of *oriC* transcripts, different fragments from the *oriC* region labelled at the 5′ end and single-stranded DNAs derived from M13mp18 or M13mp19 clones were used as

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristic	Reference
Strains		
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	37
E. coli C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	40
S. lividans 66	Wild type	16
S. lividans TK21	SLP2-SLP3 derivative of *S. lividans* 66	16
Plasmids		
pUC18	*bla*	37
pUC19	*bla*	37
Bluescript II SK(+)	pUC derivative (pMB1 replicon)	31
M13mp18	*lacZ*	37
M13mp19	*lacZ*	37
pUTH1	pUC19 derivative containing 1.1-kb *Bcl*I fragment of the Tsr^r gene	This study
pUOR2	pUC18 derivative containing *Sph*I-*Eco*RI fragment of *oriC*	42
pBOR1	Bluescript II SK(+) derivative containing *Hin*dIII-*Eco*RI fragment of pUOR2	This study
pBOR2	pBOR1 derivative containing *Eco*RI-*Bgl*II fragment of the *oriC* region	This study
pUOR5	pUC19 derivative containing *Pst*I-*Sal*I fragment of pBOR1	This study
MDA12	M13mp18 derivative containing *Alu*I-*Bam*HI fragment of the *dnaA* gene promoter region	41
MOR1	M13mp19 derivative containing *Sph*I-*Eco*RI fragment of the *oriC* region	This study
MOR2	M13mp18 derivative containing *Sph*I-*Eco*RI fragment of the *oriC* region	This study
pGEX-KG	GST gene fusion vector; *bla*	12
pGDN1	pGEX derivative containing *Bam*HI fragment of the *dnaA* gene	This study
pSOR1	Minichromosome containing *Bcl*I fragment of the *oriC* region of *S. lividans*; Tsr^r	42
pSOR2	Minichromosome containing *Sph*I-*Bgl*II fragment of the *oriC* region of *S. lividans*; Tsr^r	This study
pSOR3	Minichromosome containing *Sph*I-*Eco*RI-*Xho*II fragment of the *oriC* region of *S. lividans*; Tsr^r	This study
pSOR4	Minichromosome containing *Sph*I-*Eco*RI fragment of the *oriC* region of *S. lividans*; Tsr^r	This study
pSOR5	Minichromosome containing *Xho*II-*Eco*RI-*Bgl*II fragment of the *oriC* region of *S. lividans*; Tsr^r	This study
pIJ4083	Promoter probe plasmid (*xylE*); Tsr^r	8
pOXE1	pIJ4083 derivative containing *Eco*RI-*Hin*dIII fragment of pUOR2	This study
pOXE2	pIJ4083 derivative containing *Pst*I-*Bam*HI fragment of pUOR5	This study
pDXE12	pIJ4083 derivative containing *Alu*I-*Bam*HI fragment of the *dnaA* gene promoter region	41

hybridization probes. It was verified that a sufficient amount of probe was added to specifically tested RNAs to ensure quantitative hybridization. S1 nuclease mapping was carried out as depicted previously (41). Signal intensities were scanned by using the computer program Image Analysis, developed by Cybertech (Berlin, Germany).

Protein concentrations. The concentrations of proteins were measured by the method established by Bradford (5), with bovine serum albumin as the standard.

Cloning and purification of the glutathione *S*-transferase (GST)–DnaA fusion protein. A 723-bp *Bam*HI fragment of the *dnaA* gene (41) was cloned in frame in the pGEX-KG expression vector (12) to yield plasmid pGDN1. The orientation of this insert was verified by nucleotide sequencing. For expression, plasmid pGDN1 was transformed into *E. coli* C600. Cells were grown (600 ml) for 3 h to an optical density at 550 nm of 0.6 at 37°C in the presence of ampicillin (100 μg/ml) and induced with isopropyl-β-thiogalactoside (IPTG; 0.5 mM) at 37°C for 3 h. After centrifugation (5,000 × *g*, 4°C, 10 min), cells were suspended in PBS buffer (0.02 M phosphate buffer, 0.85% NaCl, pH 7.4) and lysed by sonification (five times, 30 s each). Inclusion bodies were sedimented by centrifugation. After dialysis against PBS buffer, the fusion protein was purified on a glutathione-Sepharose 4B column (Pharmacia) according to the supplier's manual.

Preparation of antisera. Antisera were obtained from rabbits by immunization with the purified GST-DnaA fusion protein and mixed with Freund's complete adjuvant. Serum samples were taken 10 days after the second booster injection. Cellular particles were removed by centrifugation, and antisera were stored at −20°C. Their reaction with the pure GST-DnaA fusion protein was stronger than that with GST, and they were used to detect the *S. lividans* DnaA protein in whole-cell lysates.

Immunoblot analysis of DnaA protein. *S. lividans* strains were grown in YEME medium containing 34% sucrose for 24 to 30 h at 30°C. Protoplasts were prepared by the method of Hopwood et al. (16), counted under a microscope, suspended (0.5×10^8 to 3.4×10^8 per lane) in standard loading buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and boiled for 5 min. After electrophoresis (on an SDS–10% polyacrylamide gel), separated proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell). The efficiency of transfer was controlled; the membrane was blocked with 3% bovine serum albumin and incubated with polyclonal anti-GST-DnaA fusion protein antibody. Afterwards, DnaA protein was detected with a goat anti-rabbit secondary antibody conjugated with alkaline phosphatase. The membrane was stained with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium. Cellular DnaA protein was quantified by scanning immunoblots containing different amounts of the GST-DnaA fusion protein.

RESULTS

Minimal requirements for replication. Recently, we constructed the minichromosome pSOR1 (42) which carries the origin region (*oriC*) of the *S. lividans* chromosome (a 2,584-bp fragment) and a thiostrepton resistance gene. Different fragments of pSOR1 and the Tsr^r gene were subcloned in an *E. coli* vector [pUC18, pUC19, or Bluescript II SK(+) (Table 1)] to obtain additional restriction sites. To minimize the originally cloned *oriC* region, various parts of constructs pBOR1 and pBOR2 (Table 1; Fig. 1A) and the Tsr^r gene were religated and then introduced into *S. lividans* TK21 protoplasts. Thiostrepton-resistant transformants were examined for the presence of closed circular plasmid DNA (Fig. 1B). The construct (pSOR3) which carried a deletion of the *dnaN* gene replicated as the progenitor plasmid pSOR1 did. In contrast, elimination of the *dnaN* promoter region containing a single DnaA box caused cessation of autonomous replication (construct pSOR4). The results of deletion experiments showed that the left part of the *oriC* region is also required for autonomous replication sequence activity (Fig. 1A [construct pSOR5]). Thus, the replication function can be assigned to a stretch of DNA that is longer than 623 bp but shorter than 837 bp, situated within the *Sph*I-*Eco*RI-*Xho*II fragment of pSOR3. Interestingly, the left DnaA box region is characterized by the presence of a short T-rich stretch of DNA (42). The minichromosome pSOR3 was lost from progeny in the absence of thiostrepton. To determine its copy number under selection pressure, total DNA was isolated from several pSOR3-containing transformants, cleaved with *Sal*I, and hybridized with the *Sph*I-*Eco*RI fragment from the origin region (Fig. 1C). Since the size of linearized pSOR3 differed from that of the chromosomal

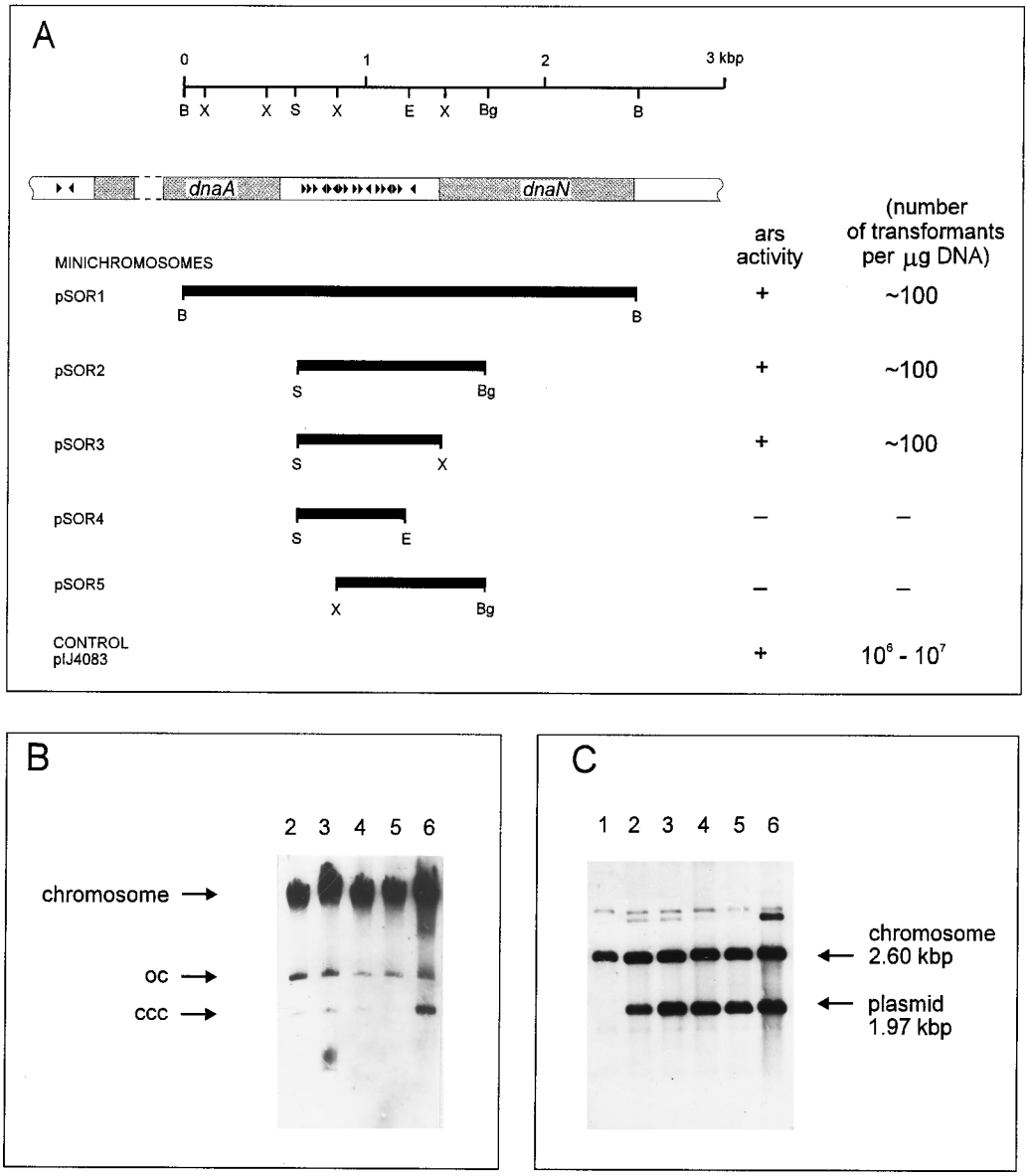

FIG. 1. Characteristics of constructed minichromosomes. (A) Restriction maps and characteristics of the various constructs. Triangles represent DnaA boxes. The presence of an autonomous replication sequence (ars+) or the lack of replication (ars−) is indicated. Restriction sites are as follows: B, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; S, *Sph*I; X, *Xho*II. (B) Identification of extrachromosomal pSOR3. The open (oc) and closed circular (ccc) forms of pSOR3 were separated from sheared chromosomal fragments by agarose gel electrophoresis. The *Sph*I-*Eco*RI *oriC* fragment was used as the probe for Southern hybridization. Lanes 2 through 6, DNA preparations from independent transformants. (C) Estimation of the copy number of pSOR3. Total DNA was digested with *Sal*I and after electrophoresis, the fragments were transferred to a nylon membrane and hybridized with a labelled *Sph*I-*Eco*RI *oriC* fragment. Lane 1, total DNA from *S. lividans* TK21; lanes 2 through 6, total DNA from independent transformants containing pSOR3.

*Sal*I fragment carrying the *oriC* region, the relative quantities of both fragments were determined simultaneously. About one pSOR3 copy per chromosome was found.

Transcriptional analysis of the *dnaN* gene. The transcriptional start site of the *dnaN* gene was determined by primer extension with reverse transcriptase, total RNA, and an oligonucleotide primer corresponding to the N terminus of the deduced DnaN protein (Fig. 2). The two identified transcriptional start sites are separated by 54 bp and correspond to positions −108 and −163 upstream of the translation start (Fig. 2). Identical results were obtained by S1 nuclease mapping (data not shown). Two promoters (P1 and P2) have been deduced, and the sequences of their predicted −35 and −10 regions (Fig. 2) correspond to class I *Streptomyces* promoters (34). It is interesting that the deduced P1 promoter region contains a DnaA box motif. Densitometric studies indicated that the transcripts corresponding to P1 were about four times less abundant than the shorter ones initiated under the control of P2.

Identification of promoter activity within the intergenic region. The *Sph*I-*Eco*RI fragment of the *oriC* region was cloned into pUC18 and Bluescript II SK(+) (Table 1). S1 nuclease mapping was carried out with *Sph*I-*Eco*RI fragments, labelled at the 5′ end of the *Eco*RI or *Sph*I site, respectively, and with total RNA isolated from the rapidly growing mycelia of *S. lividans*. Four main transcripts and several less abundant transcripts were identified with the probe labelled at the 5′ end of the *Eco*RI site (Fig. 3A, lane 1). No signals were observed after

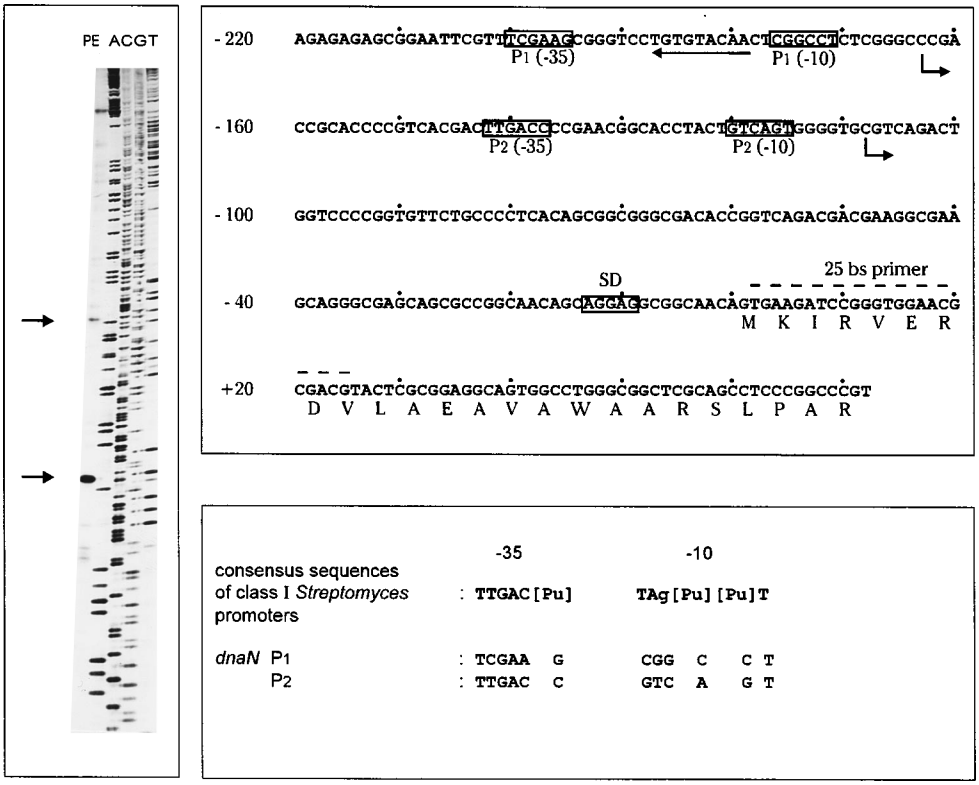

FIG. 2. Transcriptional start sites of the *dnaN* gene. (Left) Primer extension (PE) with oligonucleotides complementary to the first 25 nucleotides of the coding region was performed with 100 μg of RNA (see Materials and Methods). The two transcriptional start sites are indicated by arrows. Lanes A, C, G, and T, sequencing reactions used as molecular weight markers. (Upper right) Regulatory region of the *dnaN* gene. Deduced P1 and P2 promoters (boxes), the DnaA box motif (←), and transcriptional initiation sites (↳) are marked. SD, Shine-Dalgarno sequence.

hybridization with the probe labelled at the 5′ end of the *Sph*I site. To detect any possible internal transcript(s) within the analyzed region, the *Sph*I-*Eco*RI fragment was cloned in two orientations in M13mp18 and M13mp19. Labelled, single-stranded DNAs derived from the resulting recombinant phages (MOR1 and MOR2 [Table 1]) were used for S1 nuclease mapping studies. Hybridization with the probe derived from MOR1 resulted in S1-protected fragments which were identical to those obtained with the probe labelled at the 5′ end of *Eco*RI. One leftward transcript was identified with the probe derived from MOR2 (Fig. 3A, lane 3).

To test the in vivo activities of these deduced promoters, the *Sph*I-*Eco*RI fragment was cloned in both orientations into the promoter probe vector pIJ4083 carrying the promoterless *xylE* reporter gene (8). Thiostrepton-resistant transformants of *S. lividans* contained the expected hybrid plasmids pOXE1 and pOXE2 (Table 1). These transformants grew about two times less rapidly than did the control strains. Plasmids pOXE1 and pOXE2 were unstable and were lost from progeny in the absence of thiostrepton. Colonies which contained pOXE1 were yellow after exposure to catechol, and those carrying pOXE2 remained white. To quantify the activities of analyzed *xylE* fusions, the catechol dioxygenase activities of several strains growing in liquid cultures were compared. A transformant with the construct pDXE12 (containing a *dnaA* promoter region in front of the *xylE* gene [41]) was used as a positive control. *S. lividans* mycelia harboring pDXE12 or pOXE1 expressed inducible levels of catechol dioxygenase activity (Fig. 3C). The copy number of pOXE1 (as well as that of pOXE2) was approximately two times lower than the copy number of pIJ4083 and pDXE12 (Fig. 3D). Cultures of cells containing pOXE2 showed a low background level of expression (Fig. 3C). The data suggest the presence of a termination signal for transcription within the analyzed region of pOXE2.

Abundance of DnaA protein. The level of DnaA protein in *S. lividans* was determined with antibodies. As a prerequisite, the *Bam*HI fragment containing a truncated *dnaA* gene (see below) was subcloned in the *E. coli* expression vector pGEX-KG (Table 1) under the control of the P_{tac} promoter. The resulting construct, pGDN1, contained a fusion between the GST gene and a portion of the *dnaA* gene encoding 241 amino acids (no. 37 to 278). A transformant of *E. coli* C600 induced with IPTG synthesized large quantities of the GST-DnaA fusion, with the expected size of 54 kDa (Fig. 4A), as inclusion bodies. After solubilization, this protein was purified by affinity chromatography and used to raise antibodies. They reacted more strongly to the GST-DnaA fusion protein than they did to pure GST (data not shown) and thus were suitable for the following studies.

Mycelia of *S. lividans* harvested at the exponential-growth phase were developed into protoplasts, and cytoplasmic proteins were separated by SDS-PAGE and analyzed for reactivity with these antibodies (see Materials and Methods). A 73-kDa *S. lividans* protein (corresponding in size to the deduced *dnaA* gene product) reacted with these antibodies (Fig. 4B). By using various, defined concentrations of GST-DnaA protein as controls (see Materials and Methods) (Fig. 4B), the amount of DnaA protein was estimated to be 0.013 to 0.040% of total cellular protein from several batches of *S. lividans* protoplasts.

Effect of additional DnaA boxes. Plasmid pOXE1 contains

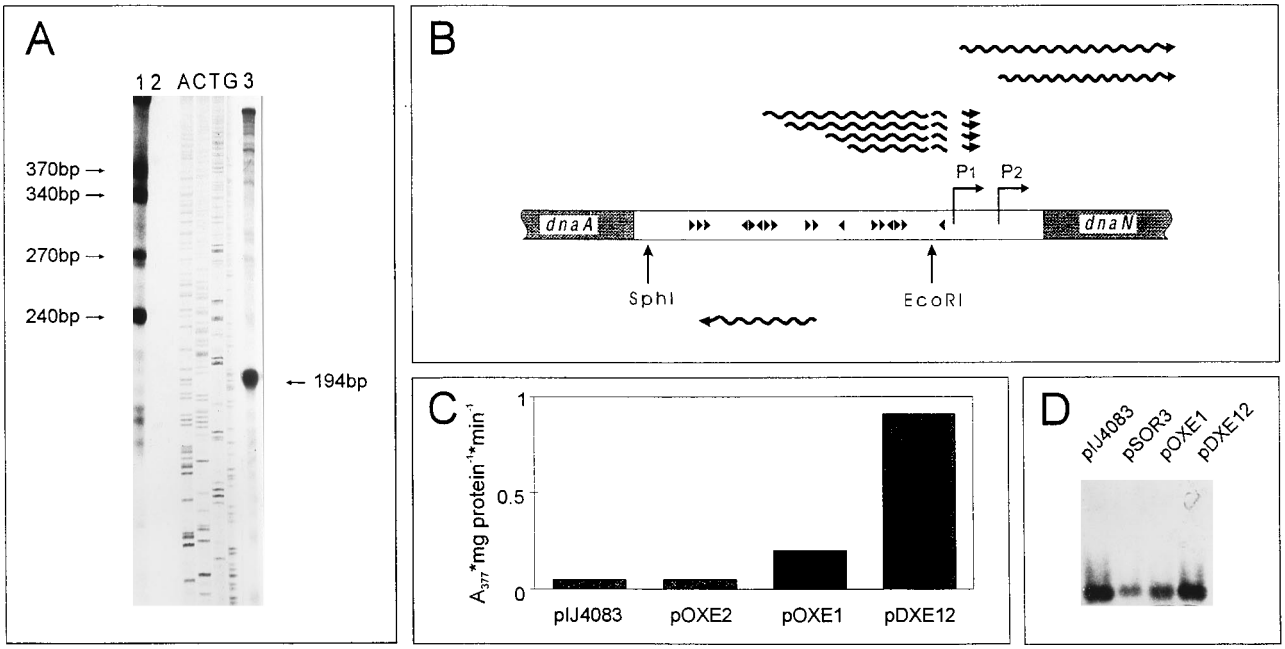

FIG. 3. Promoter activities in the *oriC* region of *S. lividans*. (A) Identification of transcripts within the *oriC* region by S1 nuclease mapping. Reactions were performed with 150 μg of total RNA (lanes 1 and 3) or 100 μg of tRNA (lane 2) and with the *Sph*I-*Eco*RI fragment labelled at the 5′ end of the *Eco*RI site (lanes 1 and 2) or single-stranded DNA derived from MOR2 (lane 3). Lanes A, C, G, and T, sequencing reactions used as molecular size markers. (B) Structural organization of the *S. lividans oriC* region. RNA transcripts (⇝), promoters (P), and DnaA boxes (◀) are marked. (C) Catechol dioxygenase activities of *S. lividans* containing various *xylE* fusions. (D) Copy numbers of various constructs containing DnaA boxes. Total DNA (2 μg) was digested with *Bcl*I, and after electrophoresis, the fragments were transferred to a nylon membrane and hybridized with the labelled Tsr^r probe (1.1-kb fragment coding for the Tsr^r gene).

16 of the 17 DnaA boxes from the *oriC* region (Table 1), and its copy number, approximately two per chromosome, is two times higher than the copy number of pSOR3 (Fig. 3D). The transcription level of the *dnaA* gene was analyzed by S1 nuclease mapping with an *Alu*I-*Bam*HI fragment from the *dnaA* promoter region subcloned into the M13mp18 phage (MDA12 [Table 1]) and with the universal primer as well as total RNA isolated from rapidly growing mycelia from TK21 and TK21(pOXE1). Each S1-protected fragment (Fig. 5A) had the expected size of 225 bases (41). As determined by densitometric scanning, the levels of corresponding transcripts from TK21(pOXE1) were about four times higher than those from TK21 mycelia. Correspondingly, the amount of the 73-kDa protein reacting with antibodies (raised against the GST-DnaA protein) was also about four times higher in rapidly grown mycelia from TK21(pOXE1) than that in those from TK21 (Fig. 5B).

DISCUSSION

Deletion analysis of a previously constructed pSOR1 minichromosome (42) revealed that a stretch of DNA which is longer than 623 bp but shorter than 837 bp is necessary for autonomous replication. All 17 DnaA boxes are located within

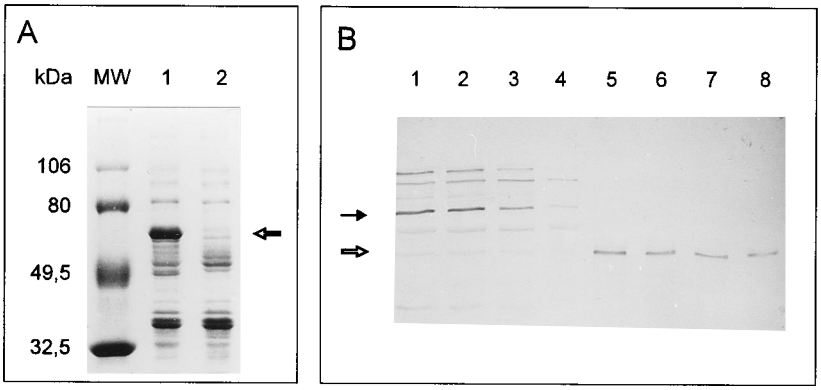

FIG. 4. Synthesis of the GST-DnaA fusion protein and detection of DnaA protein. (A) Proteins from *E. coli* C600(pGDN1) induced with IPTG (0.5 mM; lane 1) or uninduced (lane 2) were separated by SDS-PAGE. Lane MW, molecular mass markers. (B) Cellular abundance of DnaA protein in *S. lividans* TK21. Total proteins from protoplasts were separated by SDS-PAGE and tested for cross-reactions with the antibodies raised against the GST-DnaA fusion protein. Lanes 1 through 4, 3.4×10^8, 2.6×10^8, 1.7×10^8, and 0.86×10^8 protoplasts, respectively. Lanes 5 through 8, 80, 70, 60, and 50 ng of purified GST-DnaA fusion protein, respectively. The positions of the GST-DnaA fusion protein and DnaA protein are indicated by open and solid arrows, respectively.

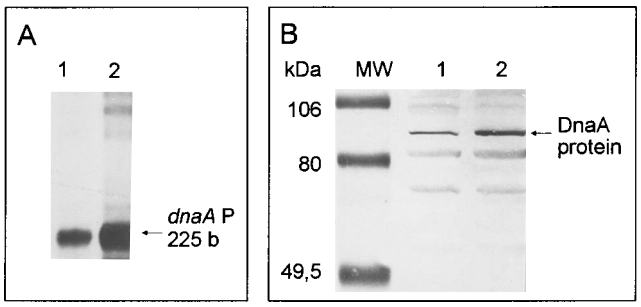

FIG. 5. Expression of the *dnaA* gene. (A) S1 nuclease mapping was performed with 100 μg of RNA isolated from rapidly grown mycelia. *dnaA* P, *dnaA* promoter; b, bases. (B) DnaA protein was detected by immunostaining (see Materials and Methods). Total protein corresponding to 5×10^7 protoplasts was loaded in each lane. Lanes: 1, *S. lividans* TK21; 2, *S. lividans* TK21(pOXE1); MW, molecular mass markers.

this region. The *oriC* region (245 bp) of *E. coli* (26) is considerably smaller and is located about 42 kb upstream of the *dnaA* gene (35). The *Bacillus subtilis* chromosome harbors two nontranslatable regions of 545 bp (*oriC*I; eight DnaA boxes) and 191 bp (*oriC*II; three DnaA boxes) which are required for autonomous replication and separated by the *dnaA* gene (1,388 bp) (22). Deletion analyses of *B. subtilis oriC* plasmids showed that the distance between the *oriC*I and *oriC*II parts can be substantially decreased (to 274 bp so far). The replication origins of *Pseudomonas putida* and *M. luteus* were found between the *dnaA* and *rpmH* genes (24, 25); the latter encodes the ribosomal protein L34.

The *E. coli oriC* region includes a high number of GATC motifs which are methylated by Dam methyltransferase. Upon replication, DNA becomes temporarily hemimethylated. It is assumed that the methylation status provides a monitor of newly replicated DNA for the cell (23). Within the *oriC* region of *S. lividans*, few GATC motifs are present. As yet, it is not known whether the *Streptomyces oriC* region contains other methylation motifs.

Similar to those of *B. subtilis* (22), the copy numbers of *S. lividans* minichromosomes are very low. Minichromosomes and plasmids containing 16 DnaA boxes from the *oriC* region of *S. lividans* are unstable and tend to be lost or integrated into chromosomal DNA. These facts are very likely due to competition among the same replicons for DnaA protein. Thus, as in *B. subtilis* (22), the initiation of *oriC* replication appears to be tightly controlled.

Two DnaA boxes were found in front of the *S. lividans dnaA* gene (41). The introduction of a plasmid containing additional DnaA boxes led to a significant increase in transcripts of the *dnaA* gene and an enhanced level of DnaA protein. This result is probably due to a titration effect by additional DnaA motifs. Our current investigation supports this interpretation; purified DnaA protein binds to the *dnaA* promoter region in vitro. Thus, as in *E. coli* (2, 6, 20) and *B. subtilis* (39), the *S. lividans dnaA* gene seems to be autoregulated by its own product. However, in *P. putida*, expression of the *dnaA* gene is apparently not autoregulated (17).

The amount of DnaA protein in rapidly growing mycelia from *S. lividans* was estimated to be 0.013 to 0.040% of total cellular protein; this corresponds to the quantities found in *E. coli* (13) and *B. subtilis* (39).

In the course of vegetative growth of *S. lividans* mycelia, the *dnaN* gene is predominantly transcribed from promoter P2. Since a DnaA box is situated in the vicinity of P1, one could speculate that transcription of the *dnaN* gene from promoter P1 is dependent on the level of DnaA protein in the course of the cell cycle.

In addition to DnaA protein, the initiation of replication in *E. coli* requires the action of RNA polymerase (33). Four promoters were identified in the *oriC* region of *E. coli* between the *gidA* and *mioC* genes (1, 21, 30). In *S. lividans*, several stop codons are located in the region between the *dnaA* and *dnaN* genes. Within all possible reading frames of this region, about 64% of the codons contain a G or C in the third position. In contrast, 93% of *Streptomyces* genes (4) contain codons with a G or C in the third position. Several transcripts (one leftward and four rightward) were discovered in this nontranslatable region by S1 nuclease mapping. The promoter activities of the rightward transcripts were confirmed in vivo by creating a transcriptional fusion with the promoterless *xylE* gene and assaying the catechol dioxygenase activities. Among possible promoter sequences in this region, only one (which shows similarities to the consensus sequence of class I *Streptomyces* promoters) corresponds to the shortest rightward transcript (−35, GTGACG; −10, TCGAGT) (34). As promoters in streptomycetes often diverge from the consensus motif, the presence of additional ones cannot be excluded. The leftward transcript is probably terminated within the analyzed region; thus, it does not allow expression of the reporter gene (*xylE*). Further subcloning is required to delete any putative transcript endpoint(s).

The *S. lividans oriC* region does not contain the three 13-bp AT-rich repeats (42) present in the left part of the *E. coli oriC* region (19). However, shorter AT-rich sequences are distributed among the DnaA boxes of the *S. lividans oriC* region. Therefore, it would be interesting to know if as in *E. coli*, the *Streptomyces* RNA polymerase modulates the initiation of replication by altering the topology of the *oriC* region.

Recently, Heintz et al. (15) suggested that eucaryotic origins are multipartite regulatory elements which resemble promoter activity. The participation of transcription factors in regulating the replication of eucaryotic chromosomes may provide a mechanism for integrating origin selection with the cell cycle-dependent transcription program. Thus, it would be interesting to compare the transcriptional and translational activities of the *oriC* region in the course of different development stages of streptomycetes.

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