Bidirectional Replication from an Internal Origin in a Linear Streptomyces Plasmid

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Commonly, linear replicons that have protein covalently attached to 5′ DNA termini replicate by protein-primed, strand-displacing, continuous synthesis of full-length strands. The synthesis of DNA in pSLA2, a 17-kilobase linear plasmid of *Streptomyces rochei* containing 5′ terminal protein, occurs bidirectionally from an internally located replication origin. The replication intermediates are linear duplex molecules that have recessed (~280 nucleotides) 5′ ends rather than full-length single strands. The 3′ overhangs may serve as templates for the non-displacing synthesis of the lagging strand terminus primed by the covalently attached 5′ DNA binding protein.

Although most plasmids of prokaryotic or eukaryotic cells exist in nature as DNA circles, some replicate as linear DNA (*1*, *2*). The telomeres of certain linear extrachromosomal replicons contain a single-strand loop that links inverted repeat sequences at the ends of duplex DNA. These replicons include *Escherichia coli* prophage N15, plasmids of the spirochete *Borrelia*, and the pox viruses of mammalian cells (*2*). Other linear replicons contain protein attached covalently to the 5′ end of each strand. Molecules of this type (for example, *Bacillus* phage ϕ29, *E. coli* phage PRD1, and the adenoviruses) have been identified in a variety of bacteria (*3*) as well as in the cytoplasm and organelles of animal and plant cells (*4*). Extensive studies of DNA replication in ϕ29 and adenovirus indicate that the protein-primed synthesis of a full-length DNA strand proceeds continuously and processively from the telomere by a mechanism that displaces the corresponding strand of the DNA duplex (*3*).

Linear plasmids of the Gram-positive, filamentous, spore-forming bacterial genus *Streptomyces* range in size from 12 to several hundred kilobase (kb) pairs (*5*–*7*). Derivatives of pSCL, a linear plasmid of *Streptomyces clavuligerous*, can replicate as circular DNA molecules when the telomeres are removed and the resulting ends are ligated (*8*). This discovery led us to investigate the replication mode of a more stably maintained *Streptomyces* linear plasmid, pSLA2, which like pSCL and other linear replicons of *Streptomyces* has protein covalently attached to its 5′ DNA ends. We report here that synthesis of the pSLA2 linear genome occurs by bidirectional replication extending outward toward the ends of the plasmid from a centrally located origin, rather than by the strand-displacing mechanism observed for other linear replicons containing 5′ DNA binding proteins.

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Transformants of *S. lividans* that received circularized pSLA2 ligated to a selectable marker gene contained a high number of copies of the circular extrachromosomal replicon (Fig. 1), indicating that like pSCL (*8*), pSLA2 includes within it a site capable of promoting replication of the plasmid in a circular form. Repeat transformation of *S. lividans* by pCIR1 (Fig. 1) occurred at an efficiency of 10^6 transformants per microgram of plasmid DNA, which is comparable to the transformation frequencies observed for other *Streptomyces* circular replicons. In the absence of selection, the pCIR1 plasmid was partitioned into *S. lividans* spores at a frequency approaching 100%. Insertion of the *E. coli*–derived pUC19 plasmid at separate sites within pCIR1 prevented replication in *S. lividans* (pCIR101 through pCIR104, Fig. 1A), indicating that multiple functions indigenous to pSLA2 are required for propagation of the circular form of the plasmid.

We used two-dimensional (2D) agarose gel electrophoresis (*9*) to determine whether replication of linear pSLA2 DNA molecules containing their native telomeres also proceeds from an internal origin. Replicating DNA typically shows one of four 2D gel patterns that depend on the location of the replication origin relative to the DNA fragment probed (*9*). Origins at the center of the fragment yield a bubble; origins outside or near the end of the fragment yield a simple Y; an off-centered origin produces an asymmetric pattern showing both a partial bubble and a Y; and a double Y results from origins occurring at both sides of the fragment. Two-dimensional gels of replicating pSLA2 DNA digested by restriction enzymes were separately probed with five restriction fragments of pSLA2 (Fig. 2): Fragment I (the 7.5-kb Bcl I fragment) showed an asymmetric bubble (Fig. 2C), indicating that a replication origin is located within this fragment. Transition of the bubble to a Y occurred about midway through its length, suggesting that the origin is located between the center of the fragment and one of its ends. Fragment II showed a simple Y pattern, indicating that the origin is outside, or at the end of, this fragment. These 2D results indicate that linear pSLA2 DNA

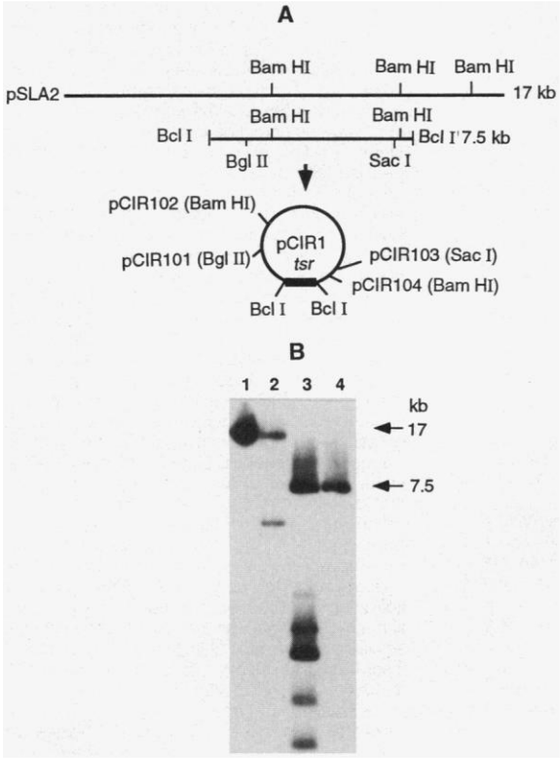

Fig. 1. (**A**) The 17-kb, linear pSLA2 DNA isolated from *S. rochei* 7434-AN4 (*15*) treated with Bcl I to remove telomeres. The resulting 7.5-kb fragment was ligated to a Bcl I–generated DNA fragment carrying the *tsr* (thiostrepton resistance) gene (*18*) and introduced by transformation into *S. lividans* TK64 (*18*). Circular plasmid DNA (pCIR1) isolated from transformants was analyzed by Southern blotting (*19*). The *E. coli* plasmids pCIR101 through pCIR104 were constructed by insertion of pUC19 DNA into pCIR1, as shown and introduced into *E. coli* JM109 (Promega). (**B**) Southern blot analysis with uncut total DNA prepared from *S. rochei* containing pSLA2 (lane 1) or *S. lividans* harboring the plasmid pCIR1 (lane 2). Lanes 3 and 4 are Bcl I–digested DNA of lanes 1 and 2, respectively. For the probe, ^{32}P-labeled pSLA2 DNA was used (*19*).

molecules contain a functional internal origin of replication and map this origin near the center of the plasmid (Fig. 2A, filled squares). The position of the origin was confirmed with other probe fragments: Fragments III and IV showed simple Y patterns (Fig. 2B), and fragment V showed a short asymmetric bubble, indicating that the replication is initiated near the end of fragment V at a site approximately equidistant from the two telomeres of the plasmid. No double Y pattern was observed for any of the fragments tested. Independent evidence that the replication of pSLA2 DNA occurs from an internal origin was provided by in situ cleavage of the DNA before electrophoresis in the second dimension (*10*): Movement of the replication fork was from right to left in fragment III and from left to right in fragment VI (Fig. 2C).

Electron microscopy and centrifugation analyses of newly synthesized adenovirus and ϕ29 DNA have shown the existence of full-length, single-strand replication intermediates (*11*, *12*). Because *Streptomyces* linear plasmids contain protein bound covalently to the 5′ terminus of each strand, it has been thought that these plasmids also replicate by a protein-primed, strand-displacing mechanism (*2*, *3*). To learn whether pSLA2 exhibits this mode of replication in addition to replication from an internal origin, we analyzed a population of pSLA2 linear plasmids isolated from log-phase cultures of *S. rochei* for the presence of single-stranded DNA. Southern (DNA) blotting of gels not subjected to denaturing conditions, which allows the detection of single-stranded DNA only, failed to show the long single-strand DNA molecules that were expected from continuous strand-displacing DNA synthesis (Fig. 3, A and B) (*3*); the gel position of full-length single strands was determined by the blotting of alkali-denatured pSLA2 DNA. However, the undenatured pSLA2 DNA showed a faint band migrating about the same position as the full-length duplex, indicating the presence of some single-strandedness in the replicating plasmid.

Given the fundamental 5′ to 3′ directionality of DNA replication, the leading strand synthesis that is initiated internally and divergently on pSLA2 must necessarily move toward the ends of the plasmid on separate DNA strands, indicating that lagging strand DNA synthesis occurs by means of discontinuous RNA-primed Okazaki fragments (*13*). This mode of DNA synthesis would leave a recessed 5′ terminus at the telomeres when the most distal RNA primer is removed. That replicative intermediates of pSLA2 DNA do have this structure was shown by Eco RI or Bam HI cleavage of pSLA2 DNA after treatment with either mung bean nuclease, which digests single-strand DNA (*14*), or *E. coli* exonuclease III (*14*). Cleavage of pSLA2 DNA by Eco RI produces a terminal fragment 0.83 kb in length, while a 1.45-kb terminal fragment is generated by Bam HI (Fig. 3C). These DNA species were unaffected by prior treatment with mung bean nuclease. However, bands observed slightly below the 0.83-kb and 1.45-kb species were reduced in size to 0.55 kb and 1.17 kb, respectively, indicating that the termini of some pSLA2 DNA molecules contain single-strand segments. The size reduction observed after mung bean nuclease digestion of these replicative intermediates shows that the single-strand regions are approximately 280 nucleotides in length at both telomeres. Because the faster migrating bands were resistant to exonuclease III, which acts on blunt-ended or recessed 3′ ends but not on projecting 3′ ends (*14*), we conclude that the pSLA2 telomeres contain 3′ overhangs.

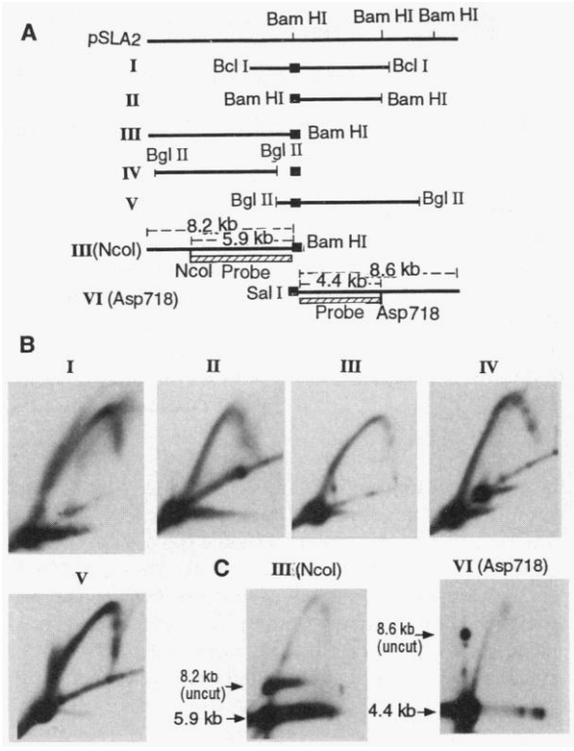

Fig. 2. (**A**) Locations of the six (I to VI) DNA fragments of pSLA2 used for 2D gel analysis (*9*). (**B**) Two-dimensional gel patterns with fragments I to V as probe. DNA from *S. rochei* was isolated from log-phase cultures and purified by BND-cellulose chromatography (*20*). Electrophoresis in the first dimension was carried out at 0.6 V/cm for 25 to 30 hours in a 0.35% tris-acetate EDTA (TAE) agarose gel. The second dimension used a 1.2% TAE agarose gel containing ethidium bromide (0.3 μg/ml); electrophoresis was at 2.5 V/cm for 12 to 16 hours. After electrophoresis, gels were blotted and hybridized individually with ^{32}P-labeled fragments I to V (*18*). (**C**) Two-dimensional gel patterns with fragments III and VI in situ digested (*10*) by Nco I and Asp 718 after first dimension. Probes used for hybridization are shown by hatching bars in (A).

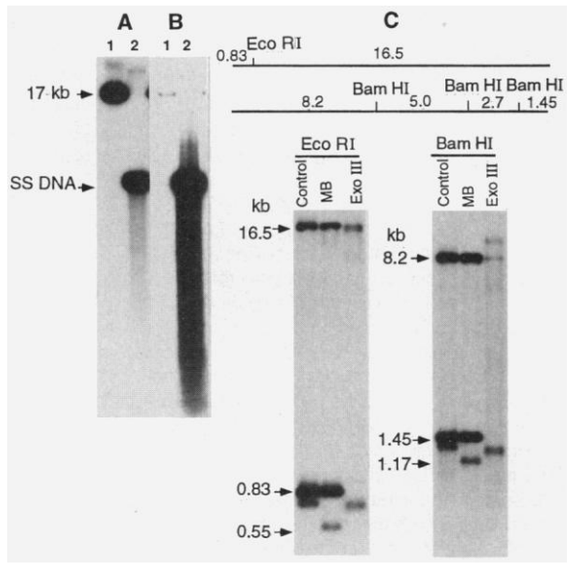

Fig. 3. (**A**) Southern blot analysis of total DNA isolated from a log-phase culture of *S. rochei* (lane 1) or pSLA2 DNA denatured by treatment with 100 mM NaOH at 37°C for 30 min (lane 2). For the probe, ^{32}P-labeled full-length pSLA2 DNA was used. Film was exposed for 2 hours. (**B**) Same experiment as in (A) except that the gel was not subjected to denaturing conditions before blotting, and film was exposed for 12 hours. (**C**) Analysis of Eco RI– or Bam HI–digested total DNA prepared from a log-phase culture of *S. rochei*. DNA was purified by BND-cellulose (*20*) and treated with the following: nothing (control), mung bean nuclease (MB), or exonuclease III (Exo III), respectively, followed by digestion with Eco RI or Bam HI (*21*). Restriction endonuclease cleavage sites for Eco RI and Bam HI and fragment sizes are indicated. The 0.83-kb Eco RI terminal DNA fragment used as a probe detects inverted repeat sequences (*5*) at both pSLA2 telomeres and minor bands of variable length produced by exonuclease III digestion at the 3′ blunt ends of nonreplicating pSLA2 molecules.

These studies show that linear pSLA2 plasmids are replicated primarily from a site near the center of the plasmid and not by full-length strand displacement initiated at the telomeres. The ends of replicating pSLA2 DNA molecules contain 3′ overhangs, and thus differ from the blunt-ended telomeres found on previously identified linear replicons that use 5′ DNA binding proteins to prime DNA synthesis. We suggest that synthesis of the 5′ terminal DNA segment of the lagging strand of pSLA2 DNA is primed, on a template that consists of the 3′ overhang of the leading strand, by the protein found earlier to be covalently attached to the 5′ termini of mature pSLA2 DNA (*15*). However, DNA synthesis initiated at the telomeres of pSLA2 does not proceed continuously through the entire molecule, unlike the protein-primed DNA synthesis that occurs in adenovirus and bacteriophage ϕ29. Instead, only a 280-nucleotide single-strand segment is filled in. Whereas internally initiated DNA replication has also been observed in certain bacteriophage linear replicons (such as T7), completion of the terminal portion of the lagging strand is accomplished by redundancy of the telomeric sequence (*16*) rather than by protein-primed DNA synthesis.

The chromosomes of several *Streptomyces* species are linear (*17*). As the inverted repeat sequences at the telomeres of the *S. lividans* chromosome are highly similar to those of pSLA2, pSCL, and other *Streptomyces* linear plasmids, we speculate that the replication mechanism found for pSLA2 may be used more generally for DNA synthesis in *Streptomyces*.

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21. About 2 μg of benzoylated naphthoylated DEAE (BND)–cellulose–purified DNA was incubated with 20 units of mung bean nuclease (Life Technologies, Grand Island, NY) in 20 μl of solution containing 10 mM sodium acetate (pH 5.0), 50 mM NaCl, 0.1 mM zinc acetate and 1 mM L-cysteine at 37°C for 15 min; or with 100 units exonuclease (New England Biolabs) in 66 mM tris (pH 8.0) and 0.66 mM $MgCl_2$ at 37°C for 60 min. The DNA treated was extracted once with saturated phenol and precipitated by ethanol before the Eco RI or Bam HI digestion.

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Ribosomal Heterogeneity from Chromatin Diminution in *Ascaris lumbricoides*

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The genome of *Ascaris lumbricoides* encodes both germline- and soma-specific proteins homologous to the eukaryotic small ribosomal protein (Rp) S19. The two *Ascaris* homologs differ by 24 amino acid substitutions and are both components of the small ribosomal subunits. In oocytes, the germline RpS19 homolog (RpS19G) predominates. During chromatin diminution, however, the gene is eliminated from all presomatic cells, and RpS19G is replaced by the product of the somatic gene (RpS19S). Chromatin diminution in *A. lumbricoides* causes a change in the protein composition of ribosomes during development and represents an alternative means of gene regulation.

Chromatin diminution in nematodes, which discard genomic DNA from all presomatic cells during early embryonic development, represents an exception to the DNA "constancy" rule. This process has been reported in about a dozen Ascaridae species (*1*, *2*), which are nematodes that parasitize vertebrates and some invertebrates (*3*). In *Ascaris lumbricoides*, the eliminated material contains large amounts of highly repetitive satellite DNA and also certain amounts of less repetitive and single-copy DNA sequences (*1*, *4*). One expelled gene (*alep-1*) encodes a putative protein (RpS19G) homologous to the small ribosomal subunit protein S19 of eukaryotes (*5*). The *alep-1* gene is transcribed in all cells of the germ line, but no *rpS19G* transcripts are found in somatic cells, from which the gene is absent (*5*).

The 148–amino acid *rpS19G* gene product is a component of the small subunit of the *Ascaris* oocyte ribosomes (Fig. 1A), as we predicted from its sequence. Although the protein was absent from the ribosomes of muscle cells, antiserum to RpS19G (*6*) detected another protein that had a slightly reduced mobility on gels. A weak band of similar mobility was also extracted from oocyte ribosomes (Fig. 1A). The almost identical molecular size and similar antigenic properties suggested that the larger protein is an RpS19 isoform, which may be encoded by a gene that is not eliminated by chromatin diminution and is present in both the soma and germ line.

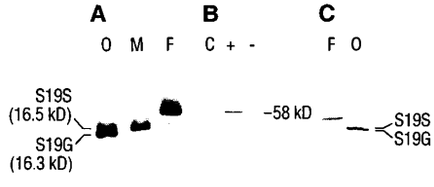

Fig. 1. Distribution of *A. lumbricoides* RpS19G and RpS19S. (**A**) Protein immunoblot with ~4 μg per lane of sucrose-purified small ribosomal subunits from *A. lumbricoides* oocytes (O) and muscles (M) and an aliquot of a bacterial protein extract containing the RpS19G fusion protein (F). The concentration of the small ribosomal subunits was standardized on standard two-dimensional gels (*17*). (**B**) Protein immunoblot of the RpS19S fusion protein. RpS19G antibodies detected the 58-kD RpS19S fusion protein (*18*) in IPTG-induced TB1 cells containing the fusion vector (+), but not in noninduced cells (−) or in induced cells containing the empty expression vector pMalC2 (C). (**C**) Total protein extract from *Ascaris* oocytes (O) and an aliquot of the RpS19G fusion protein as control (F). Isolation and purification of small ribosomal subunits from *A. lumbricoides* oviducts or muscle tissues were performed as described (*19*). Protein extracts were analyzed by electrophoresis with a 16%T (total concentration of monmers), 6%C (cross-linking agent) polyacrylamide gel. Protein transfer and immunoblotting with the serum to a bacterially expressed RpS19G fusion protein (*6*) were done as described (*20*). A secondary antibody, an alkaline phosphatase–conjugated goat antibody to rabbit immunoglobulin (Dako), was used.