JOURNAL OF BACTERIOLOGY, Apr. 1991, p. 2378–2384
0021-9193/91/072378-07$02.00/0

Vol. 173, No. 7

# Transcription of the Stability Operon of IncFII Plasmid NR1

YOU-NONG MIN, AKIRA TABUCHI, DAVID D. WOMBLE,* AND ROBERT H. ROWND

*Department of Cell, Molecular, and Structural Biology, The Medical and Dental Schools, Northwestern University, Chicago, Illinois 60611*

Received 13 September 1990/Accepted 23 January 1991

**The stability (*stb*) locus of IncFII plasmid NR1 is composed of an essential *cis*-acting DNA site located upstream from two tandem genes that encode essential stability proteins. The *stb* locus was found to be transcribed from a promoter site just upstream from the first gene, *stbA*. This promoter was active for transcription both in vivo and in vitro and was located within the region that includes the essential *cis*-acting site. Transcripts initiated from this site were approximately 1,500 to 1,600 nucleotides in length. Northern (RNA) blot analysis indicated that the transcripts traversed both *stbA* and the downstream gene, *stbB*. Mutants from which the promoter had been deleted failed to produce detectable transcripts from either *stbA* or *stbB*. Transcription of a third open reading frame, *stbC*, which is contained within the *stbB* gene in the opposite DNA strand, could not be detected. For a mutant in which a transposon had been inserted in *stbA*, no transcription of *stbB* was detected. After deletion of most of the transposon, which left behind a 35-bp frameshift insertion in *stbA*, transcription of *stbB* was restored, although the insertion still had a polar effect on *stbB* function. The rate of in vivo transcription of the *stb* locus was measured by pulse-labeling of RNA followed by quantitative RNA-DNA hybridization. Mutants deleted of *stbB* had an approximately 10-fold increase in the rate of transcription, whereas those deleted of the promoter region had at least a 10-fold reduction in transcription rate. The half-life of *stb* mRNA was approximately 2 min. These data suggest that *stbA* and *stbB* are cotranscribed as an operon that may be autoregulated.**

NR1, a self-transmissible 95-kbp antibiotic resistance plasmid of the FII incompatibility group (31), has a low number of copies per cell and yet is inherited by virtually every cell descended from an original plasmid-bearing cell in culture. The stability (*stb*) locus of NR1 is essential for this process of stable inheritance (16, 23). Because the stability function is independent of plasmid replication or copy number control, *stb* is thought to participate in the partitioning of plasmid molecules to daughter cells during cell division. Mutants of NR1 that lack *stb* function are unstable, so that plasmid-free cells are segregated at a rate consistent with random distribution of the plasmid copies at cell division (16).

The *stb* locus of NR1 (Fig. 1), which is probably equivalent to the *parA*$^+$ locus of the related IncFII plasmid R1 (9), is contained within a 1.7-kb region bounded by *Nae*I and *Taq*I restriction sites (27) and is located approximately at NR1 coordinates 24.5 to 26.1 kb (31). It is composed of an essential *cis*-acting DNA site followed by two genes, *stbA* and *stbB* (18, 27). These genes encode *trans*-acting proteins of 36,000 and 13,000 Da, respectively, that are essential for the stability function (27). The stop codon of *stbA* overlaps the start codon of *stbB* (27). A putative transcription promoter, $P_{AB}$, lies upstream of *stbA* in the region that contains the *cis*-acting site (18, 27). A third open reading frame, *stbC*, is contained within *stbB* and is encoded in the opposite DNA strand (18, 27). No protein product from *stbC* has been detected (27), and no function has yet been found for *stbC*. The overall organization of *stb* may appear to be basically similar to that of the *sop* locus of plasmid F (3, 21) and the *par* locus of plasmid prophage P1 (1, 3, 4), which are essential for the stability of those plasmids (2, 20). However, the essential *cis*-acting sites, or partition sites, of those plasmids lie downstream from their two essential tandem genes, and there are other differences in the behaviors of these various stability loci that suggest that they may function by somewhat different mechanisms (2, 5, 20).

Mutations in either *stbA* or *stbB* can be complemented in *trans* (18, 27). Plasmids that retain the *cis*-acting site but lack *stbA* and *stbB* can be stabilized in *trans* by a plasmid that can supply both StbA and StbB, whereas mutants that lack the site cannot be complemented (18, 27). A transposon insertion or a small frameshift insertion in *stbA* have polar effects on *stbB*, in that these mutants require a *trans* supply of both StbA and StbB for restoration of stability (18). Here we report that the *stbA* and *stbB* genes are cotranscribed from the same promoter, $P_{AB}$, and that the transcription is most likely autoregulated. Since the promoter is located within the region of the *cis*-acting site, that site may function in both autoregulation and plasmid partitioning.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** *Escherichia coli* K-12 strains KP245 (15) and JM105 (33) were used for all experiments. 2YT broth (17) was used for most cultures, including those for plasmid DNA and RNA isolation. 1xA minimal medium (17) supplemented with appropriate amino acids was used for pulse-labeling of RNA and plasmid copy number measurements. Antibiotics were included in the media to select cells containing various antibiotic resistance plasmids. Ingredients for media were obtained from Difco Laboratories and Sigma Chemical Company. Growth was monitored by turbidity measurements at 650 nm with a Gilford model 260 spectrophotometer.

**Plasmids.** Most of the plasmids used in these studies (Table 1) are derivatives of NR1, a natural isolate (19, 24, 31), and recombinant vector plasmids pUC8, pUC9 (29), and pSF2124 (26), which is a ColE1 derivative that contains a Tn*3* insertion and single sites for cleavage by *Eco*RI and *Sma*I. pRR720 is a stable 24.6-kb miniplasmid derivative that

* Corresponding author.

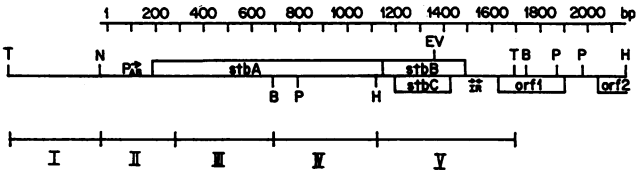


FIG. 1. Map of NR1 stability locus. The *stb* locus is contained within a 1.7-kb *Nae*I (N)-*Taq*I (T) restriction fragment (27). Genes *stbA* and *stbB* encode proteins of 36,000 and 13,000 Da, respectively (27). There is an essential *cis*-acting site located in the upstream region near transcription promoter $P_{AB}$ (18). A third open reading frame of unknown significance, *stbC*, is in the DNA sequence of the opposite strand within *stbB*. An inverted repeat (IR) that is not essential for *stb* function is located downstream from *stbB* (27). Two other open reading frames, orf1 and orf2, are encoded in the opposite DNA strand and are located outside the stability locus. Of unknown function, orf1 encodes a protein of 11,000 Da (27). Other restriction sites are *Bgl*II (B), *Eco*RV (EV), *Hinc*II (and *Hpa*I) (H), and *Pst*I (P). The numbers above the map indicate positions in the *stb* DNA sequence (27). The line below the map indicates the DNA fragments used as probes in various experiments: probe I, *Taq*I-*Nae*I; probe II, *Nae*I-*Bst*uI; probe III, *Bst*uI-*Bgl*II; probe IV, *Bgl*II-*Hinc*II; probe V, *Hinc*II-*Taq*I.

TABLE 1. Plasmids used in this study

| Plasmid | Description | Reference(s) |
|---|---|---|
| pRR720 | NR1-derived miniplasmid, *stb*$^+$ | 22, 27 |
| pMR1 | Unstable mutant (*stbA*::Tn*1732*) of pRR720 | 18, 27 |
| pFR12 | Unstable mutant (*stbB* nonsense) of pRR720 | 18, 27 |
| pMR13 | Unstable mutant (*stbA* frameshift) of pRR720 | 18, 27 |
| pMR21 | Unstable mutant (*stbA* missense) of pRR720 | 18, 27 |
| pMR37 | Unstable mutant (deletion of $P_{AB}$) of pRR720 | 18, 27 |
| pMR81 | Unstable mutant (*Bgl*II deletion) of pRR720 | 18, 27 |
| pSF2124 | ColE1-derived cloning vector | 26 |
| pMR420 | pSF2124 containing *stb* from pRR720 | 18, 27 |
| pMR412 | pSF2124 containing *stb* from pFR12 | 18, 27 |
| pMR421 | pSF2124 containing *stb* from pMR21 | 18, 27 |
| pMR437 | pSF2124 containing *stb* from pMR37 | 18, 27 |
| pMR481 | pSF2124 containing *stb* from pMR81 | 18, 27 |
| pUC8, pUC9 | pMB1-derived cloning vectors | 29 |
| pFR820 | pUC8 containing *stb* from pRR720 | 18, 27 |
| pMR920 | pUC9 containing *stb* from pRR720 | 18, 27 |
| pFR812 | pUC8 containing *stb* from pFR12 | 18, 27 |
| pMR921 | pUC9 containing *stb* from pMR21 | 18, 27 |
| pMR837 | pUC8 containing *stb* from pMR37 | 18, 27 |
| pMR835 | *Eco*RV deletion of pMR837 | 18, 37 |
| pMR881 | pUC8 containing *stb* from pMR81 | 18, 37 |
| pMR938 | *Hpa*I deletion of pMR920 | 18, 27 |
| pIBI30, pIBI31 | Cloning vectors with T7 and T3 promoters | |

contains the *cat* gene and wild-type *repA* and *stb* functions of NR1 (22, 27). The *stb* locus is contained within a 6.4-kb *Sma*I-*Eco*RI fragment that has been cloned in either the moderate-copy-number vector pSF2124 or the high-copy-number pUC vectors (27).

Various unstable mutants of pRR720 that contain point, deletion, or insertion mutations in *stb* have been described (18, 27). pMR1 contains Tn*1732* (28) inserted at bp 610 in *stbA*. All but 35 bp of the transposon can be conveniently deleted by partial digestion with *Eco*RI followed by self-ligation (28). pMR13 was constructed from pMR1 in this manner, so that it contains a 35-bp insertion within *stbA* as described previously (18, 27). pMR21 contains a missense substitution at bp 775 in *stbA*. pFR12 contains an ocher substitution at bp 1439 in *stbB*. This mutation lies outside the coding sequence for *stbC*. pMR37 contains a deletion entering from the left and proceeding through bp 287. Therefore, promoter $P_{AB}$ and the 5′ end of *stbA* have been deleted from pMR37. pMR835 is a pUC8 clone of the *stb* locus from pMR37 that contains an additional deletion between the *Eco*RV site at position 1360 and a second *Eco*RV site located farther to the right that is not shown on the map in Fig. 1. Therefore, in addition to the deletion of $P_{AB}$, the 3′ end of *stbB* has been deleted from pMR835. pMR81 contains a deletion between the *Bgl*II sites at positions 689 and 1745; therefore, the 3′ half of *stbA* and all of *stbB* have been deleted from pMR81. pMR938 was derived from a pUC9 clone of the stability locus from which the DNA between the *Hpa*I sites at positions 1119 and 2159 had been deleted; therefore, pMR938 is missing the last seven codons of *stbA* and all of *stbB*.

**Primer extension experiments.** To determine the 5′ ends of the RNA transcripts from the *stb* locus, the methods for primer extension described elsewhere (6) were followed. Synthetic oligonucleotides of 35 bases that were complementary to the sequences near the 5′ ends of *stbA*, *stbB*, and *stbC* were obtained from the biotechnology support center of Northwestern University and used as primers. The 5′ ends of the primers were labeled with [$\gamma$-$^{32}$P]ATP (New England Nuclear) and T4 polynucleotide kinase (Pharmacia). The primers were hybridized to the RNA samples (see below) and extended with avian myeloblastosis virus reverse transcriptase (Bethesda Research Laboratories). Placental RNase inhibitor was obtained from Promega Biotech. Most other chemicals and reagents used in this and the other procedures described below were obtained from Fisher or Sigma. The 5′-end-labeled extension products were analyzed on polyacrylamide sequencing gels and compared with sequencing reactions prepared from the same primers and *stb* DNA by the dideoxy method of Sanger et al. (25). The products were detected by autoradiography with Kodak X-Omat AR film. Sequencing reagents and enzymes were obtained from New England BioLabs, Inc. In vivo RNA samples were obtained from cultures of cells that contained various plasmids by the method of Womble et al. (32). In vitro-generated transcripts were obtained by the method of Lau and Roberts (14). *E. coli* RNA polymerase holoenzyme was a gift from L. F. Lau.

**Northern (RNA) blot analysis.** RNA samples were subjected to Northern blot analysis by the methods described elsewhere (6) with slight modifications. The formaldehyde concentration in the agarose gels was increased fivefold, and formaldehyde was also included in the running buffer at the same concentration in order to run the electrophoresis slowly overnight for better separation. Intact RNA samples were obtained from cultures of cells containing various plasmids by the method of Glisin et al. (10) as modified by Kornblum et al. (13) or by the method of Deuschle et al. (8), with similar results. RNA samples were prepared for electrophoresis by mixing 20 μl of RNA sample with 40 μl of deionized formamide and 8 μl of 10× MOPS (morpholine-

propanesulfonic acid) running buffer (6) and heating the mixture at 65°C for 10 min, followed by chilling on ice. The denatured RNA samples were then electrophoresed in 1.2% agarose–formaldehyde gels. The RNA was transferred from the gels to supported nitrocellulose membranes (Optibind; 0.45-μm pore size; Schleicher & Schuell) and hybridized to $^{32}$P-labeled RNA or DNA probes. The hybridization products were detected by autoradiography.

The DNA restriction fragments from various regions of the *stb* locus that were used to prepare the probes are shown at the bottom of Fig. 1 and are labeled I to V. The labeled probes were prepared by two different methods. First, the restriction fragments were cloned into the pIBI30 and pIBI31 vector plasmids, which have transcription promoters for T7 and T3 RNA polymerase on either side of the polylinker cloning site (International Biotechnologies, Inc.). Strand-specific RNA probes were then prepared in vitro with either T7 or T3 RNA polymerase (International Biotechnologies, Inc.) and [α-$^{32}$P]ATP (New England Nuclear). Second, the DNA fragments were labeled by nick translation with [α-$^{32}$P]dATP (New England Nuclear). Nick translation reagents and enzymes were obtained as a kit from Bethesda Research Laboratories. The sizes of the RNA transcripts were estimated by comparison with the mobilities of RNA ladder molecular weight standards (Bethesda Research Laboratories) that were electrophoresed in the same gels. The locations of the standard bands were determined by slicing off the lanes containing the RNA ladder, staining with ethidium bromide, and photographing the gel with UV light. The RNA samples to be transferred to nitrocellulose were not stained with ethidium bromide, which improved the efficiency of transfer and hybridization.

**In vivo pulse-labeling and hybridization of RNA.** The rate of *stb* transcription in vivo was determined as described by Womble et al. (32). Exponential-phase cultures of cells containing various plasmids were pulse-labeled with [5-$^{3}$H] uridine (New England Nuclear), and RNA was isolated as described before (32). The RNA samples were subjected to quantitative RNA-DNA hybridization to determine the fraction of input RNA that bound specifically to the single-stranded probe DNA on each filter in multiple replicates. The probe DNA was obtained from various restriction fragments from the *stb* locus, such as those used for the Northern blot analysis (Fig. 1), or from clones of these fragments in phages M13mp18 and M13mp19 (33). The M13 clones, with the fragments inserted in either orientation, provided strand-specific hybridization probes that could be used to determine the direction of transcription and to distinguish between transcripts from the overlapping *stbB* and *stbC* genes.

**RNA half-life measurements.** The stability of *stb* mRNA was determined by adding rifampin (300 μg/ml) to exponential-phase cultures of cells containing *stb*$^{+}$ plasmids and collecting samples at various times thereafter. The cell samples were killed with ice and sodium azide, and the RNA in each sample was extracted and subjected to Northern blot analysis as described above. The hybridized RNA was detected by autoradiography, and the bands were quantitated with a laser densitometer. The half-life was calculated by graphing the log of the fraction of RNA remaining at each time point and fitting the points to a linear least-squares plot.

**Plasmid copy number measurements.** The relative copy numbers of various plasmids were estimated by electrophoresis of plasmid DNA from minilysates of cells on 0.8% agarose gels. The amount of cells in each minilysate was equalized by comparing the turbidity at 650 nm and adjusting the volume of culture added. Plasmid DNA was isolated by the alkaline minilysate method (7) and linearized by using appropriate restriction enzymes. The agarose gels were stained with ethidium bromide and photographed with UV light. The intensity of each plasmid band in the photographic negatives was determined by laser densitometry. The intensities were normalized to the known molecular weights of the DNA fragments in the bands and compared with the bands from pRR720, which was defined to have a relative copy number of one.

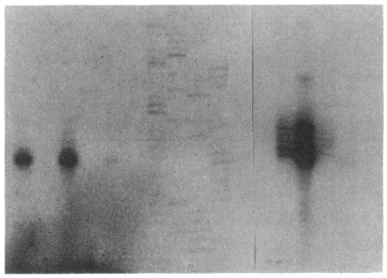


FIG. 2. Location of the 5′ end of *stb* mRNA. Primer extension was used to locate the 5′ end of RNA transcripts obtained either in vitro from transcription of *stb* DNA (lanes 1 through 5) or in vivo from cultures of cells containing plasmids (lanes 6 and 7). Templates for RNA transcription were: lanes 1 and 3, supercoiled pMR881 plasmid DNA; lane 2, purified *Sma*I-*Bgl*II restriction fragment from pMR881; lane 4, purified 1.7-kb *Nae*I-*Taq*I restriction fragment from pMR920; lane 5, purified 1.15-kb *Nae*I-*Hin*cII restriction fragment from pMR920; lane 6, pMR920; and lane 7, pMR938. The primary start site was located at bp 115 (Fig. 1) by comparison with a dideoxy sequencing ladder prepared from the same oligonucleotide primer, shown between lanes 3 and 4.

## RESULTS

**Location of transcription initiation sites within the *stb* locus in vivo and in vitro.** The 5′ ends of *stb* transcription products were examined by the primer extension method. Synthetic oligonucleotides that contained DNA sequences from near the 5′ ends of *stbA*, *stbB*, and *stbC* that were expected to be complementary to transcripts of those genes were obtained and used as primers. Transcription products were generated either in vitro with purified DNA templates and RNA polymerase or in vivo in cells containing various plasmid derivatives. With the primer from *stbA*, in vitro transcripts having a primary initiation site at bp 115 in the *stb* sequence (Fig. 1) were identified from supercoiled plasmid DNA templates (Fig. 2, lanes 1 and 3). Similarly, in vitro transcripts from various linear restriction fragments containing the *stb* region also had a primary initiation site upstream from *stbA* at position 115 (Fig. 2, lanes 2, 4, and 5). A similar initiation site at approximately position 115 was identified for the transcripts synthesized in vivo from plasmids pMR930 and pMR938 (Fig. 2, lanes 6 and 7). There were several other bands representing possible in vitro initiation sites within a few bases of position 115. It is not clear whether these represent actual minor variations in the initiation site or were produced by some variability in the length of the labeled primer. In any case, position 115 is the initiation site expected for the putative transcription promoter $P_{AB}$ originally identified from the DNA sequence (27), which we now

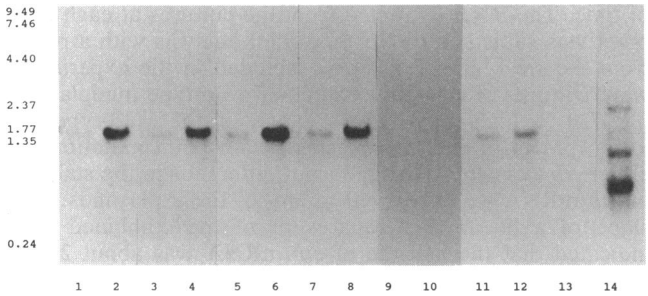


FIG. 3. Northern blot analysis of *stb* mRNA. Lanes: 1, pRR720; 2, pMR420; 3, pFR12; 4, pMR412; 5, pRR720; 6, pMR420; 7, pFR12; 8, pMR412; 9, pMR437; 10, pMR37; 11, pRR720; 12, pMR13; 13, pMR1; 14, pMR1. Hybridization was to probes IV (lanes 1 to 4), V (lanes 5 to 13), and III (lane 14). See Fig. 1 for maps of the probes. The positions of the size standards in the RNA ladder (Bethesda Research Laboratories) are indicated at the left (in kilobases).

conclude to be the actual promoter located upstream from *stbA*.

Similar experiments with a probe from the 5′ end of *stbB* revealed minor in vitro initiation sites at positions 1119, 1011, and 850 in the nucleotide sequence upstream from *stbB* and within *stbA*. Position 1119 corresponds to the putative promoter $P_B$ identified from the nucleotide sequence (27). However, all of these bands were quite weak, and no corresponding initiation sites could be identified from the in vivo-generated RNA transcripts (data not shown). We conclude that there are no significant transcription initiation sites located just upstream from *stbB* that can be detected by primer extension. In addition, no initiation sites upstream from *stbC* on the opposite DNA strand could be detected from RNA transcripts generated either in vitro or in vivo (data not shown).

**Size and composition of *stb* mRNA in vivo.** Northern blot analysis was used to investigate the size and composition of *stb* mRNA in vivo (Fig. 3). RNA isolated from cells containing various plasmids was hybridized to probes complementary to various regions of the *stb* locus (Fig. 1). A transcript that was 1,500 to 1,600 nucleotides in length was made from both wild-type and *stbB* point mutant plasmids and various clones thereof (Fig. 3, lanes 1 through 8). A similar transcript was also made by *stbA* point mutant plasmids (data not shown). This transcript hybridized to probes from *stbA* (Fig. 3, lanes 1 through 4) and *stbB* (Fig. 3, lanes 5 through 8). It also hybridized to probe II, which overlaps $P_{AB}$ and the 5′ end of *stbA*, but not to probe I, from upstream of *stbA* (data not shown). Strand-specific probes generated from the pIBI vectors and T7 or T3 RNA polymerase (see Materials and Methods) indicated that the direction of transcription was left to right, as shown in Fig. 1 (data not shown). No transcripts with the opposite polarity were detected. Assuming that the 1,500- to 1,600-base transcript has its 5′ end at position 115, as identified in Fig. 2, it is sufficient to encode both *stbA* and *stbB*.

Mutant pMR37 has had $P_{AB}$ and the 5′ end of *stbA* deleted. Northern blot analysis of RNA transcribed from pMR37 and clones derived from its *stb* locus showed that no transcripts could be detected with probes from *stbA* (data not shown) or *stbB* (Fig. 3, lanes 9 and 10). This indicated that transcription of both *stbA* and *stbB* was dependent on promoter $P_{AB}$. Plasmid pMR835 has had the 3′ end of *stbB*, distal to the *Eco*RV site, deleted in addition to the deletion of $P_{AB}$. pMR835 also produced no RNA transcripts detectable with a probe from *stbB* (data not shown).

TABLE 2. Rate of *stb* mRNA transcription in vivo

| Plasmid | Description | Relative transcription rate[a] | Relative copy no.[b] | Rate/ copy no. |
|---|---|---|---|---|
| pRR720 | Stable (wild-type *stb*) | 1.0 | 1.0 | 1.0 |
| pMR21 | *stbA* point mutant | 1.0 ± 0.2 | 1.1 ± 0.0 | 0.9 |
| pFR12 | *stbB* point mutant | 1.2 ± 0.3 | 1.1 ± 0.0 | 1.1 |
| pMR81 | *Bgl*II deletion of *stbA* and *stbB* | 8.1 ± 3.4 | 1.1 ± 0.2 | 7.4 |
| pMR37 | Deletion of $P_{AB}$ | 0.1 | | |
| pMR420 | Wild-type *stb* cloned in pSF2124 | 2.5 ± 0.2 | 3.9 ± 0.1 | 0.6 |
| pMR421 | *stbA* mutant cloned in pSF2124 | 2.8 ± 1.2 | 4.1 ± 0.2 | 0.7 |
| pMR412 | *stbB* mutant cloned in pSF2124 | 2.6 ± 0.3 | 3.7 ± 0.4 | 0.7 |
| pMR481 | *Bgl*II deletion cloned in pSF2124 | 32 ± 2 | 1.8 ± 0.1 | 18 |

[a] The rate of *stb* mRNA transcription in vivo was measured by pulse-labeling RNA in cultures of cells containing the indicated plasmids, followed by quantitative RNA-DNA hybridization to probe III DNA (Fig. 1). The rates determined from multiple replicates were averaged and normalized to that of wild-type pRR720 (set at 1).

[b] The relative copy number of each plasmid except pMR37 was determined by the alkaline minilysate method. Averages of two or more determinations were normalized to the value obtained for pRR720 (set at 1).

Mutant pMR1 contains an insertion of Tn*1732* in *stbA*. Most of the transposon was deleted between the *Eco*RI sites near each of its ends to produce mutant pMR13, which retains a 35-bp frameshift insertion in *stbA*. Both mutations behave as if polar on *stbB*, since both StbA and StbB proteins must be provided in *trans* for complementation of these mutants to stability (18). No RNA transcribed from pMR1 could be detected with probe IV (not shown) or probe V (Fig. 3, lane 13). These probes contain DNA located downstream from the insertion site at position 610. Probe III, which is from the upstream half of *stbA*, detected large quantities of transcripts of several sizes from pMR1 (Fig. 3, lane 14). These probably represent different termination sites within Tn*1732* of transcripts initiated at $P_{AB}$. In contrast, an approximately 1,600-base *stb* transcript was made by pMR13 (Fig. 3, lane 12), indicating that removal of the transposon from *stbA* restored transcription of the downstream *stbB* gene. It is possible that the transcript from pMR13 was 35 bases longer than normal owing to the 35-bp insertion in *stbA*.

**Rate of *stb* mRNA transcription in vivo.** To measure the rate of *stb* mRNA transcription in vivo, cultures of cells containing various plasmid derivatives were pulse-labeled with [5-$^3$H]uridine. The RNA was extracted from the cultures and quantitatively hybridized to determine the fraction of total labeled RNA that was complementary to various single-stranded DNA probes from the *stb* region (Table 2). Strand-specific hybridization probes indicated that transcription was from left to right as shown in Fig. 1. The rates of *stb* transcription from plasmids with point mutations in either *stbA* or *stbB* were approximately the same as that from the wild-type *stb* locus. The rate of *stb* transcription from a mutant in which *stbB* and half of *stbA* were deleted was approximately 10-fold higher than the wild-type rate. This suggested that *stb* mRNA transcription was derepressed in this mutant. The rate of *stb* mRNA transcription measured for a mutant that had $P_{AB}$ deleted was lower by about a

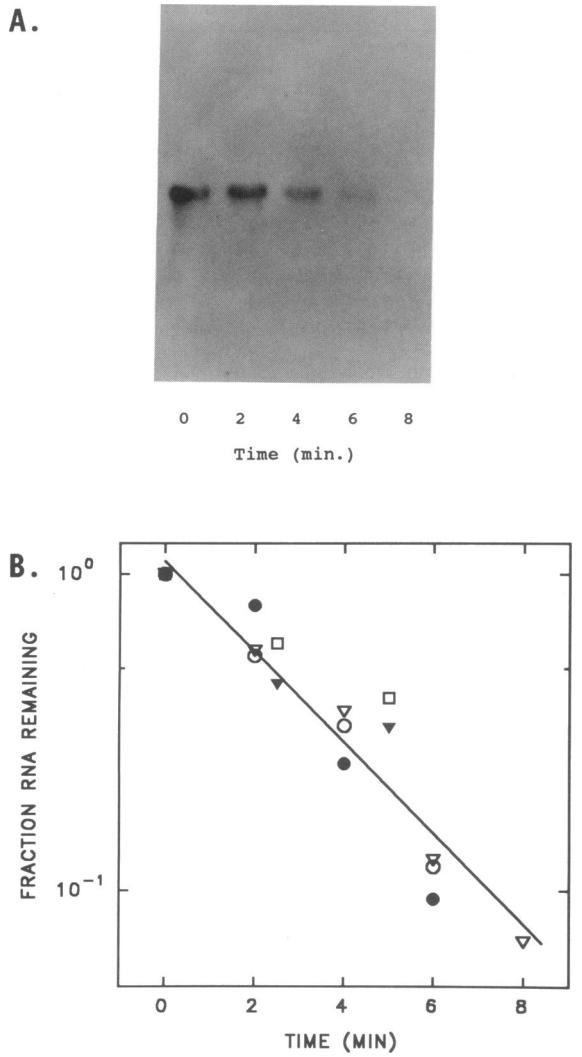


FIG. 4. Stability of *stb* mRNA. (A) RNA was isolated from cells containing plasmid pRR720 at various times after the addition of rifampin and subjected to Northern blot analysis with probe III (see Fig. 1). The autoradiograms were scanned with a laser densitometer to determine the fraction of RNA remaining at each time point. (B) Linear least-squares plot of data from five experiments similar to that shown in panel A. The plasmids examined were pRR720 (○, □), pMR21 (●), pFR12 (▽), and pMR920 (▼). From the slope of the line, the half-life of the *stb* mRNA was calculated to be 2.1 min.

factor of 10. Although the relative copy numbers of the pSF2124 clones were approximately 4-fold higher than those of miniplasmid pRR720 and its mutants (Table 2), the rates of *stb* mRNA transcription of the clones with wild-type, *stbA*, or *stbB* loci were only about 2.5-fold higher. This could indicate transcriptional autoregulation in response to copy number. In contrast, the high rates of *stb* mRNA transcription from the deletion mutants pMR81 and pMR481 suggest a lack of regulation in the absence of active *stbA* and *stbB* genes.

**Half-life of *stb* mRNA in vivo.** The stability of *stb* mRNA in vivo was determined by adding rifampin to cultures to prevent further initiation of RNA transcription and then monitoring the fraction of *stb* mRNA remaining as a function of time. The RNA extracted from the cultures at each time point was subjected to Northern blot analysis with a probe from the *stb* region (Fig. 4A). Included in the experiment were cultures of cells that contained wild-type miniplasmid pRR720, a high-copy-number clone of the wild-type *stb* locus, pMR920, and also plasmids with point mutations in either *stbA* or *stbB*. No significant difference in the stability of *stb* mRNA was observed in any of these plasmids. The slope of a linear least-squares fit of the combined data indicated that the half-life of *stb* mRNA was about 2 min (Fig. 4B).

## DISCUSSION

Although there are three open reading frames within the DNA sequence of the minimal *stb* locus, protein products were previously detected only for *stbA* and *stbB*, not for *stbC* (27). Examination of the DNA sequence revealed potential transcription promoter sequences in front of each of the three possible genes. The results of primer extension experiments reported here indicated that promoter $P_{AB}$, located just upstream from *stbA*, was active for transcription both in vivo and in vitro and on both circular and linear DNA templates. The primary initiation site was at position 115 in the *stb* DNA sequence (Fig. 2). Whereas a weak transcription initiation site at position 1119, which would correspond to promoter $P_B$ predicted from the DNA sequence, was detected from the in vitro transcripts, none was detected from the in vivo transcripts. No transcription initiation sites upstream of *stbC* were detected from either in vivo or in vitro transcripts. $P_{AB}$ therefore seems to be the only significant transcription promoter within the *stb* locus.

Northern blot analysis of *stb* RNA indicated that the transcript initiated at $P_{AB}$ traversed both *stbA* and *stbB* and was approximately 1,500 to 1,600 nucleotides in length (Fig. 3). The half-life of the *stbAB* mRNA was approximately 2 min, which is about average for *E. coli* mRNA (12, 30). When $P_{AB}$ was deleted, transcripts from neither *stbA* nor *stbB* could be detected, indicating that transcription of both genes was dependent on $P_{AB}$. In addition, no *stb* transcripts were detected from a mutant, pMR835, in which both $P_{AB}$ and the 3′ half of *stbB* were deleted. This indicated that the lack of transcription in vivo from putative promoter $P_B$ was not the result of repression by either the StbA or StbB protein, since both were inactivated by the deletions in pMR835.

Insertion of the 7-kb transposon Tn*1732* in *stbA* also abolished transcription of *stbB*. Therefore, the polarity on *stbB* of this insertion in pMR1 is most likely a result of direct blockage of *stbB* transcription, which is dependent on $P_{AB}$, as a result of transcription termination within the transposon. The larger amount of transcription detected with the probe having homology upstream from the insertion site could indicate that transcription from $P_{AB}$ was derepressed in mutant pMR1. Similar evidence for autoregulation of the related *parA* locus of IncFII plasmid R1 has also been reported (9). Mutant pMR13 contains a frameshift mutation in *stbA* that was a consequence of deletion of most of the transposon from pMR1 (18, 27). Northern blot analysis of the RNA transcribed from pMR13 indicated that transcription of *stbB* was restored in this mutant. Since the *stbB* gene is transcribed in pMR13, the apparent polarity on *stbB* of the frameshift in *stbA* requires some other explanation. The translation stop codon of *stbA* overlaps the translation

initiation codon of *stbB* (27). Perhaps translation of the *stbB* portion of the mRNA is coupled to translation of *stbA* (11), and the coupling is disrupted by the frameshift insertion in pMR13.

No transcripts from the possible gene *stbC* predicted from the DNA sequence were detected by Northern blotting. Together with the above data, this suggests that *stbC* may not be a functional gene, since neither transcripts nor protein products could be detected. However, no mutants that have alterations in the *stbC* sequence without simultaneous alterations in *stbB* have yet been examined. Therefore, the question of the significance of *stbC* requires additional investigation.

Examination of the rates of in vivo transcription of the various mutants revealed several interesting aspects of the control of *stb* expression (Table 2). First, transcription was again shown to be dependent on $P_{AB}$. Second, deletion of *stbB* and half of *stbA* resulted in a 10-fold increase in the transcription rate of the remaining region upstream from the deletion. Together with our other data, this suggests that the *stb* operon is autoregulated by either StbB or StbA or both. Other experiments with gene fusions have shown that the StbB protein by itself has repressor activity (27a). The reduced rates of transcription on a per-plasmid basis of the higher-copy-number clones (Table 2) could indicate a gene dosage response to autorepression, as would be expected in most cases of autoregulated operons. Since the *cis*-acting site and promoter $P_{AB}$ are located in the same region just upstream from *stbA*, this may indicate that this region of the DNA serves dual functions, regulation and stability. Although the partition loci of plasmids F and P1 also appear to be composed of two genes transcribed from single autoregulated promoters, the *cis*-acting sites seem to be located downstream from the genes for those plasmids (5, 20). It will be interesting to examine the binding properties of the StbA and StbB proteins and to determine their sites of action both in regulation of expression of the *stb* operon and in the partitioning of plasmid molecules to daughter cells during cell division.

## ACKNOWLEDGMENTS

We thank Lester F. Lau for the gift of RNA polymerase and Xinnian Dong for advice on in vitro transcription experiments.

This work was supported in part by Public Health Service research grant GM-30731 from the National Institutes of Health.

## REFERENCES

1. **Abeles, A. L., S. A. Friedman, and S. J. Austin.** 1985. Partition of unit-copy miniplasmids to daughter cells. III. The DNA sequence and functional organization of the P1 partition region. J. Mol. Biol. **185**:261–272.
2. **Austin, S. J.** 1988. Plasmid partition. Plasmid **20**:1–9.
3. **Austin, S., and A. Abeles.** 1983. Partition of unit-copy miniplasmids to daughter cells. I. P1 and F miniplasmids contain discrete, interchangeable sequences sufficient to promote equipartition. J. Mol. Biol. **169**:353–372.
4. **Austin, S., and A. Abeles.** 1983. Partition of unit-copy miniplasmids to daughter cells. II. The partition region of miniplasmid P1 encodes an essential protein and a centromere-like site at which it acts. J. Mol. Biol. **169**:373–387.
5. **Austin, S., and K. Nordstrom.** 1990. Partition-mediated incompatibility of bacterial plasmids. Cell **60**:351–354.
6. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
7. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
8. **Deuschle, U., W. Kammerer, R. Gentz, and H. Bujard.** 1986. Promoters of *Escherichia coli*: a hierarchy of in vivo strength indicates alternate structures. EMBO J. **5**:2987–2994.
9. **Gerdes, K., and S. Molin.** 1986. Partitioning of plasmid R1: structural and functional analysis of the *parA* locus. J. Mol. Biol. **190**:269–279.
10. **Glisin, V., R. Crkvenjakov, and C. Byus.** 1974. Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry **13**: 2633–2637.
11. **Gold, L.** 1988. Posttranscriptional regulatory mechanisms in *Escherichia coli*. Annu. Rev. Biochem. **57**:199–233.
12. **Kennell, D., and H. Riezman.** 1977. Transcription and translation initiation frequencies of the *Escherichia coli lac* operon. J. Mol. Biol. **114**:1–21.
13. **Kornblum, J. S., S. J. Projan, S. L. Moghazeh, and R. P. Novick.** 1988. A rapid method to quantitate non-labeled RNA species in bacterial cells. Gene **63**:75–85.
14. **Lau, L. F., and J. W. Roberts.** 1985. ρ-dependent transcription termination at λtR1 requires upstream sequences. J. Biol. Chem. **260**:574–584.
15. **Miki, T., A. M. Easton, and R. H. Rownd.** 1978. Mapping of the resistance genes of the R plasmid NR1. Mol. Gen. Genet. **158**:217–224.
16. **Miki, T., A. M. Easton, and R. H. Rownd.** 1980. Cloning of replication, incompatibility, and stability functions of R plasmid NR1. J. Bacteriol. **141**:87–99.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Min, Y. N., A. Tabuchi, Y. L. Fan, D. D. Womble, and R. H. Rownd.** 1988. Complementation of mutants of the stability locus of IncFII plasmid NR1. Essential functions of the trans-acting *stbA* and *stbB* gene products. J. Mol. Biol. **204**:345–356.
19. **Nakaya, R., A. Nakamura, and Y. Murata.** 1960. Resistance transfer agents in *Shigella*. Biochem. Biophys. Res. Commun. **3**:654–659.
20. **Nordstrom, K., and S. J. Austin.** 1989. Mechanisms that contribute to the stable segregation of plasmids. Annu. Rev. Genet. **23**:37–69.
21. **Ogura, T., and S. Hiraga.** 1983. Partition mechanism of F plasmid: two plasmid gene-encoded products and a cis-acting region are involved in partition. Cell **32**:351–360.
22. **Peterson, B. C., and R. H. Rownd.** 1985. Recombination sites in plasmid drug resistance gene amplification. J. Bacteriol. **164**: 1359–1361.
23. **Rownd, R. H., A. M. Easton, C. R. Barton, D. D. Womble, J. McKell, P. Sampathkumar, and V. Luckow.** 1980. Replication, incompatibility, and stability functions of R plasmid NR1, p. 311–334. *In* B. Alberts (ed.), Mechanistic studies of DNA replication and recombination. Academic Press, Inc., New York.
24. **Rownd, R., R. Nakaya, and A. Nakamura.** 1966. Molecular nature of the drug-resistance factors of the Enterobacteriaceae. J. Mol. Biol. **17**:376–393.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
26. **So, M., R. Gill, and S. Falkow.** 1975. The generation of a ColE1 $Ap^r$ cloning vehicle which allows detection of inserted DNA fragments. Mol. Gen. Genet. **142**:239–249.
27. **Tabuchi, A., Y. N. Min, C. K. Kim, Y. L. Fan, D. D. Womble, and R. H. Rownd.** 1988. Genetic organization and nucleotide sequence of the stability locus of IncFII plasmid NR1. J. Mol. Biol. **202**:511–525.

27a. **Tabuchi, A., D. Womble, and R. Rownd.** Unpublished data.

28. **Ubben, D., and R. Schmitt.** 1986. Tn*1721* derivatives for transposon mutagenesis, restriction mapping and nucleotide sequence analysis. Gene **41**:145–152.
29. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
30. **von Gabain, A., J. G. Belasco, J. L. Schottel, A. C. Y. Chang,**

**and S. N. Cohen.** 1983. Decay of mRNA in *Escherichia coli*: investigation of the fate of specific segments of transcripts. Proc. Natl. Acad. Sci. USA **80**:653–657.

31. **Womble, D. D., and R. H. Rownd.** 1988. Genetic and physical map of plasmid NR1: comparison with other IncFII antibiotic resistance plasmids. Microbiol. Rev. **52**:433–451.
32. **Womble, D. D., P. Sampathkumar, A. M. Easton, V. A. Luckow, and R. H. Rownd.** 1985. Transcription of the replication control region of the IncFII R-plasmid NR1 *in vitro* and *in vivo*. J. Mol. Biol. **181**:395–410.
33. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.