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In vivo production of a stable single-stranded cDNA in *Saccharomyces cerevisiae* by means of a bacterial retron

(multicopy single-stranded DNA/branched RNA/retroelement/*Escherichia coli*/reverse transcriptase)

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ABSTRACT **Gram-negative bacteria such as *Myxococcus xanthus*, *Stigmatella aurantiaca*, and *Escherichia coli* contain retroelements called retrons. Retrons consist of the *msr–msd* region and the gene for reverse transcriptase (RT), which are essential for the production of the branched RNA-linked msDNA (multicopy single-stranded DNA). In this study, we attempted to produce msDNA in the yeast *Saccharomyces cerevisiae*. Retron Ec67 from *E. coli*, which is responsible for the production of msDNA-Ec67, was cloned under the *GAL10* promoter in a 2-μm-based plasmid. msDNA thus produced was detected by extending the 3′ end of the msDNA by avian myeloblastosis virus RT. This yielded a main product of 117 nucleotides. Treatment of this product with RNase A resulted in a DNA of 105 nucleotides. These results are in good agreement with the structure of msDNA-Ec67. The production of msDNA-Ec67 was further confirmed by Southern blot hybridization. The msDNA production was dependent upon the bacterial RT gene in the clone and was increased severalfold when the RT gene of retron Ec67 was placed in front of the *msr–msd* region. The potential of msDNA as a eukaryotic vector producing a stable single-stranded DNA as well as RNA is discussed.**

The production of single-stranded cDNA by reverse transcriptase (RT) using an RNA transcript as a template is an obligatory step for RT-mediated transposition of retroelements (see refs. 1 and 2 for reviews). Such transposition is involved in integration of retroviruses into mammalian genomes, production of infectious retroviruses from proviruses integrated into genomes, retrotransposition of retroelements, and formation of pseudogenes in eukaryotic cells. However, single-stranded cDNAs produced *in vivo* by RT have never been directly detected, probably because of their very low amounts in the cell.

Gram-negative bacteria such as *Myxococcus xanthus*, *Stigmatella aurantiaca*, and *Escherichia coli* contain a retroelement called a retron (see refs. 3 and 4 for reviews). A retron is a small genetic element, 1.3–2.5 kilobases (kb) in length, consisting of the *msr–msd* region followed by the gene for RT. These bacterial RTs have been shown to be evolutionarily related to retroviral RTs. The primary transcript from the *msr–msd* region serves not only as a template but also as a primer to produce a single-stranded cDNA called msDNA (multicopy single-stranded DNA). msDNA production is primed from an internal rG residue of the RNA transcript, using its 2′-OH group. Thus msDNA is branched out from this rG residue by a 2′,5′-phosphodiester linkage. msDNA is a very stable cDNA in bacterial cells, existing at a level of several hundred molecules per cell. Its stability is attributed at least partially to its branched structure at the 5′ end as well as to its 3′ end forming a DNA·RNA hybrid with the 3′ end of the template RNA molecule.

In the present study we attempted to produce msDNA in *Saccharomyces cerevisiae* by introducing one bacterial retron, Ec67 from *E. coli*, under the transcriptional control of the *GAL10* promoter. A unique product due to retron Ec67 was identified on the basis of its size before and after RNase A treatment and by Southern blot analysis. The msDNA production was found to be absolutely dependent upon the RT gene in the retron and was increased by a few fold when the RT gene was moved in front of the *msr–msd* region.

MATERIALS AND METHODS

Yeast Strains, Media, and Growth Conditions. Yeast strain SP1 (**a** *ura3 leu2 trp1 his3 ade8 can*^{r} *gal2*) was used. Cells were grown in YPD medium (1% yeast extract/2% Bacto-Peptone/2% glucose). For screening transformants of YEp521 and its derivatives a minimal medium was used (5), supplemented with all nutrients required but leucine. For galactose induction, 0.15 ml of the precultured cells in the minimal medium containing 2% glucose was added to 40 ml of the minimal medium supplemented with 2% galactose instead of glucose. The cells were grown at 30°C until late logarithmic phase. Yeast transformations were carried out by the lithium acetate method (6). Transformation of yeast cells was confirmed as follows: the plasmid prepared from yeast transformants was transformed into *E. coli* DH5 [F$^-$ *endA1 recA1 hsdR17* (rk$^-$mk$^+$) *supE44 thi-1, gyrA96, relA1*] and the plasmid prepared from DH5 cells was subsequently characterized. Plasmid DNA from yeast cells was prepared according to Hoffman and Winston (7).

Plasmids. YEp52 (8) was used to construct plasmids for expression of msDNA in yeast; this plasmid contains the ColE1 origin of replication, a promoter of the *GAL10* gene, *LEU2*, the 2-μm-circle origin of replication, and the 2-μm-circle *REP3* locus. Retron Ec67 was prepared from plasmid pCl-1BPv4 in which the 4-kb *Bal* I–*Pvu* II fragment (DNA fragment from the *Bal* I site to the second *Pvu* II site from the left end of the map described in figure 5 of ref. 9) was cloned into the *Hin*cII site of pUC9. *E. coli* harboring this plasmid produces msDNA-Ec67. pCl-1EP5a and pCl-1EP5c contain the 5-kb *Pst* I(a)–*Eco*RI fragment encompassing the entire 4-kb *Bal* I–*Pvu* II sequence in pCl-1BPv4 (see figure 5 of ref. 9) in pBR322 and pUC9, respectively.

Plasmid Construction. Plasmid YEp521 was constructed by introducing the multiple cloning sites of pUC19 (10) into YEp52 (8), which was designed to obtain high-level, inducible expression of a cloned gene under the *GAL10* promoter in yeast. The DNA fragment containing the pUC19 multiple cloning site was isolated by digestion of pUC19 with *Eco*RI, the cleaved ends were filled in with the Klenow fragment of DNA polymerase I, and the fragment was digested with

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Abbreviations: AMV, avian myeloblastosis virus; msDNA, multicopy single-stranded DNA; RT, reverse transcriptase.

*Hin*dIII. The resulting 54-base-pair (bp) fragment was cloned into YEp52 by replacing a fragment between the *Bcl* I (filled in with the Klenow fragment) and *Hin*dIII sites, resulting in YEp521. YEp521, thus constructed, contains the multiple cloning sites from pUC19, except for *Eco*RI, downstream of the *GAL10* promoter. The 4-kb *Hin*dIII–*Bam*HI fragment from pCl-1BPv4 was cloned into the *Hin*dIII and *Bam*HI sites of YEp521. As a result, the *msr–msd* region and the RT gene of retron Ec67 were placed downstream of the *GAL10* promoter. This plasmid is designated YEp521-M1 (Fig. 1).

To eliminate a fragment of 242 bases upstream of *msr* that contains several ATG codons, a polymerase chain reaction (PCR) was performed using YEp521-M1 as a template with two synthetic oligonucleotides, M2-a (5′-GCAAGCTTCATAAACACGCATGT-3′) and M2-b (5′-CTGGATCCAGAAACGCATGCAGG-3′), as primers. These sequences correspond to bases 243–258 (M2-a) and bases 384–369 (M2-b) of retron Ec67 (see figure 6 of ref. 9), which flank the *msr–msd* region. The 140-bp PCR product was gel-purified and digested with *Hin*dIII and *Bam*HI. The resulting fragment was cloned into the *Hin*dIII and *Bam*HI sites of YEp521, yielding YEp521-M2. YEp521-M2 contains only the *msr–msd* region under the *GAL10* promoter.

To insert the RT gene at the *Bam*HI site of YEp521-M2, the 1.8-kb *Bam*HI fragment encompassing the RT gene was amplified by PCR using YEp521-M1 as a template and two oligonucleotide primers, M3-a (5′-CTGGATCCAAGAAGAAATGACAAAAACA-3′) and M3-b (5′-CTGGATCCTTCATTAGCTATTTAACAT-3′), which correspond to bases 409–429 and bases 2182–2163 of retron Ec67 (see figure 6 of ref. 9), respectively. The *Bam*HI sites at both ends of the fragment were created by PCR. The 1.8-kb fragment was gel-purified, digested with *Bam*HI, and cloned into the *Bam*HI site of YEp521-M2. The resulting plasmid was designated YEp521-M3. The same *Bam*HI fragment was also cloned into the *Bam*HI site of YEp521 to give plasmid YEp521-RT.

YEp521-M4 was constructed to change the order of the *msr–msd* region and the RT gene. The *msr–msd* region was amplified by PCR using the same sequence of M2-a and M2-b (see above) except that *Sma* I sites were added at their 5′ ends. Subsequently, the 140-bp *Sma* I fragment containing the *msr–msd* region was cloned into the *Sma* I site of YEp521-RT to give plasmid YEp521-M4.

Detection of msDNA. A total RNA fraction from yeast cells was prepared as described by Elder *et al.* (11), and total RNA fractions from a clinically isolated *E. coli* strain Cl-1 and from *E. coli* harboring pCl-1EP5c were prepared as described by Chomzynski and Sacchi (12). msDNA-Ec67 used for Southern blot hybridization was isolated from 20 ml of an overnight culture of CL83 harboring pCl-1EP5a (9) and was treated with RNase A.

To label msDNA with RT, the total RNA fraction prepared from 0.9 ml (for Fig. 2*A*) or 0.3 ml (for Fig. 3) of a late-logarithmic-phase culture was added to 20 μl of a reaction mixture containing 50 mM Tris·HCl (pH 8.2), 50 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 5 μCi (185 kBq) of [α-^{32}P]dATP, and 5 units of avian myeloblastosis virus (AMV) RT (Molecular Genetic Resources, Tampa, FL). The reaction mixture was incubated at 37°C for 1 hr, and an aliquot was subjected to electrophoresis in a 6% polyacrylamide/8 M urea gel (Fig. 2*A*) or a 4% polyacrylamide/8 M urea gel (Fig. 3). Another aliquot was treated with RNase A (10 μg/ml) for 10 min at 37°C and subjected to electrophoresis.

msDNA-Ec67 was also detected by Southern blot analysis (13). Total RNA from 0.6 ml of a late-logarithmic culture with or without RNase A treatment was applied to a 1.0% agarose gel with 40 mM Tris·HCl, pH 8.0/10 mM sodium acetate/2 mM EDTA. After electrophoresis, the nucleic acids were blotted from the gel to a nylon membrane filter (Pall Biodyne A transfer membrane; ICN) by capillary transfer. Hybridization was carried out in 50% (vol/vol) formamide/900 mM NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA/0.5% sodium dodecyl sulfate/5× Denhardt's solution (14) with the nick-translated 140-bp *msr–msd* region as a probe (15).

RESULTS

msDNA Production in *S. cerevisiae*. To examine whether retron Ec67 from *E. coli* can be expressed in yeast cells to

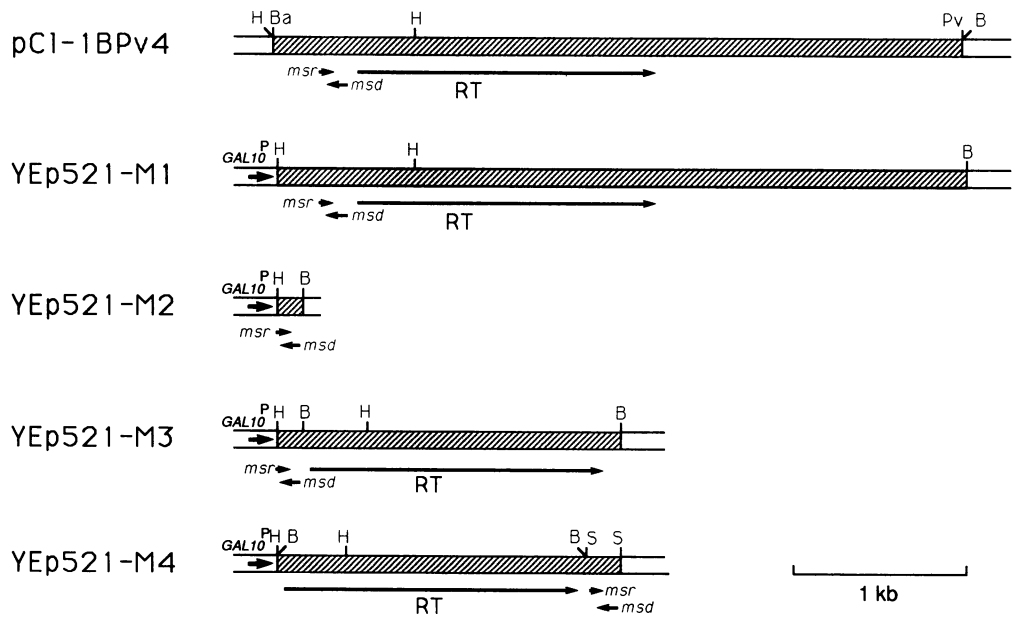

FIG. 1. Diagram of plasmids pCl-1BPv4 and YEp521-M1, -M2, -M3, and -M4. Only the regions (hatched bars) inserted in the yeast vector (YEp521) are shown. These regions contain retron Ec67, and only those restriction sites that were used for the construction of plasmids are shown (H, *Hin*dIII; Ba, *Bal* I; Pv, *Pvu* II; B, *Bam*HI; S, *Sma* I) (see *Materials and Methods*). Short arrows with *msr* or *msd* are the locations and the orientations of *msd* RNA and msDNA. Long arrows with RT represent the gene for RT and its orientation. The thick arrows represent the *GAL10* promoter (*GAL10*^{P}) and its orientation of transcription.

produce msDNA-Ec67, the 4-kb *Hin*dIII–*Bam*HI fragment was cloned downstream of the *GAL10* promoter in plasmid YEp521. Total RNA was isolated from the yeast cells harboring YEp521-M1 and was labeled with [α-^{32}P]dATP by AMV RT. The products were analyzed in a 6% polyacrylamide/urea gel (Fig. 2*A*). If msDNA exists in the total RNA fraction, AMV RT should extend the DNA strand from the 3′ end of msDNA by using *msd* RNA as a template (Fig. 2*B*). The DNA extension then terminates at the branched rG residue (9). A putative RT product from msDNA-Ec67 is considered to consist of 118 nucleotides including 67 bases from the original msDNA, 36 bases added by the RT extension at the 3′ end of the msDNA, and 15 bases from the 5′-end RNA arm of the *msd* RNA upstream of the branched rG residue (band a in Fig. 2*B*).

When the products from yeast cells harboring YEp521-M1 were analyzed by polyacrylamide/urea gel electrophoresis, three major bands appeared at positions of 117, 118, and 119

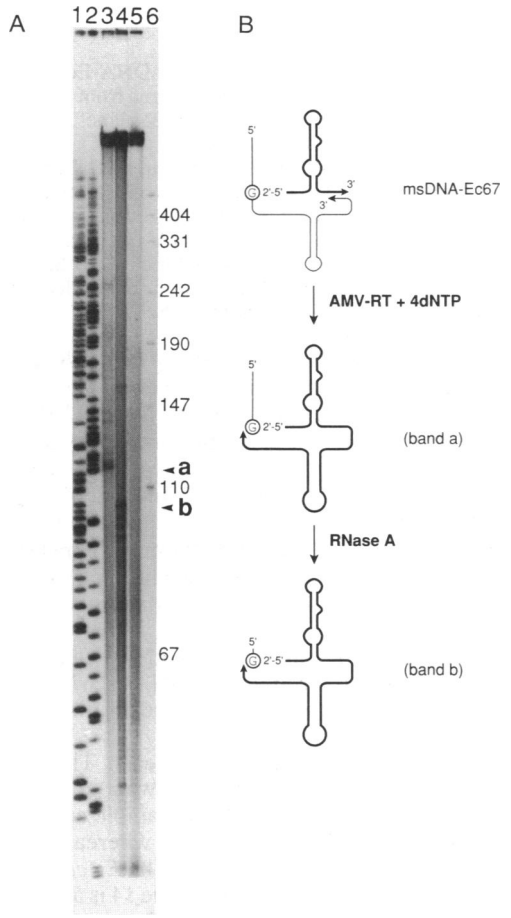

FIG. 2. Production of msDNA-Ec67 in *S. cerevisiae* harboring YEp521-M1. (*A*) Total RNA prepared from 0.9 ml of a late-logarithmic-phase culture was used for detecting msDNA with AMV RT. The RT reaction mixture was subjected to electrophoresis in a 6% polyacrylamide/urea gel. An aliquot of the reaction mixture was treated with RNase A prior to gel electrophoresis. Lanes 1 and 2 (G and C lanes, respectively) are DNA sequence ladders of pUC19 sequenced by the chain termination method (16) to provide size markers; lane 3, AMV RT products with total RNA from yeast cells harboring YEp521-M1; lane 4, same as lane 3 except that it was treated with RNase A prior to electrophoresis; lane 5, AMV RT products with total RNA from yeast cells harboring YEp521 (treated with RNase A); lane 6, *Msp* I digest of pBR322 labeled with [α-^{32}P]dCTP with the Klenow fragment of DNA polymerase I. Numbers at right indicate fragment sizes (bp) and arrowheads a and b indicate positions of msDNA. (*B*) Extension of the 3′ end of msDNA by AMV RT and RNase A treatment of its product (see text for details).

bases (arrowhead a in Fig. 2*A*, lane 3), which agree well with the expected size described above. When the RT-extended products were treated with RNase A before gel electrophoresis, band a disappeared and a new band appeared at position b (Fig. 2*A*, lane 4) corresponding to a 104-base DNA fragment. Note that this band was not detected in the RT-extended products of total RNA from yeast cells harboring YEp521 (Fig. 2*A*, lane 5). Since RNase A treatment is considered to remove 13 bases from the 5′ end of the *msd* RNA on the basis of the structure of msDNA-Ec67 (17), the RNase A product can be calculated to consist of 105 bases (103 bases from DNA plus 2 bases from RNA), which again agrees well with the size of the band-b product (Fig. 2*B*).

After RNase A treatment, several other bands appeared at positions lower than band b (Fig. 2*A*, lane 4). It has been shown that in the case of cell-free msDNA synthesis in *M. xanthus* (18), DNA extension by RT was partial, leaving DNA fragments of various sizes that were linked to RNA molecules of compensatory sizes. When these products were analyzed without RNase A treatment by polyacrylamide/urea gel electrophoresis, they migrated at the same position as the fully extended product. However, when the products were treated with RNase A before electrophoresis, they formed a "ladder" in a polyacrylamide/urea gel (18).

Effect of Gene Arrangement in Retron Ec67 on msDNA Production. A ribosome scans through an mRNA from its 5′ end and initiates translation from the first AUG codon of the mRNA in eukaryotic cells (19). In YEp521-M1, there are 417 bp from the 5′-end *Hin*dIII site to the initiation codon for RT (see figure 6 of ref. 9). In this region, there are 12 AUG codons that are considered to reduce the production of RT. Therefore, we first attempted to delete the 240-bp sequence upstream of the *msr* gene (from the leftmost *Hin*dIII site to immediately upstream of *msr* of YEp522-M1 in Fig. 1). For this purpose, the 140-bp *msr–msd* fragment including 5 extra bases upstream of *msr* and 18 extra bases at the 3′ end of the *msr–msd* region (upstream of *msd*) was amplified by PCR. Similarly, the 1.8-kb RT gene was also amplified by PCR (including 8 bases upstream of the initiation codon of the RT gene and 4 bases downstream of the termination codon). These fragments were cloned into the *Hin*dIII and *Bam*HI sites of YEp521, yielding YEp521-M3 (Fig. 1). Thus the 5′ noncoding region was shortened from 242 to 6 bp.

However, since the *msr–msd* region still contained three AUG codons, we further constructed YEp521-M4, in which the *msr–msd* region was inserted after the RT gene (Fig. 1). In YEp521-M4, there is one AUG codon between the leftmost *Hin*dIII site and the *Bam*HI site that exists in the multiple cloning sites of pUC19 (10). This AUG codon is terminated by a termination codon, UAG, after five codons. The initiation codon for the RT gene is placed six codons after the termination codon in the same reading frame. The production of msDNA-Ec67 in yeast cells transformed with YEp521-M1, -M3, and -M4 was examined by RT extension reaction. Total RNA fractions isolated from late-logarithmic cells harboring YEp521, -M1, -M2, -M3, -M4, and -RT were examined by the AMV RT extension method as performed in Fig. 2*A*. The products were analyzed in a 4% acrylamide/8 M urea gel with or without RNase treatment (Fig. 3). As in the case of Fig. 2*A*, YEp521-M1 RNA produced a band (Fig. 3, lane 3) at the same position as msDNA-Ec67 isolated from *E. coli* strain Cl-1 (arrowhead a, lane 13); see the structure shown for band a in Fig. 2*B*). In samples treated with RNase A, two new bands (lane 4) appeared again at the same positions as bands b and c of msDNA-Ec67 (lane 14). Band b is the fully extended DNA fragment corresponding to band b in Fig. 2*B*. Band c is considered to be a product resulting from the extension from the 3′ end of *msd* RNA, using msDNA as a template, and thus to consist of a single-stranded DNA of ≈60 bases.

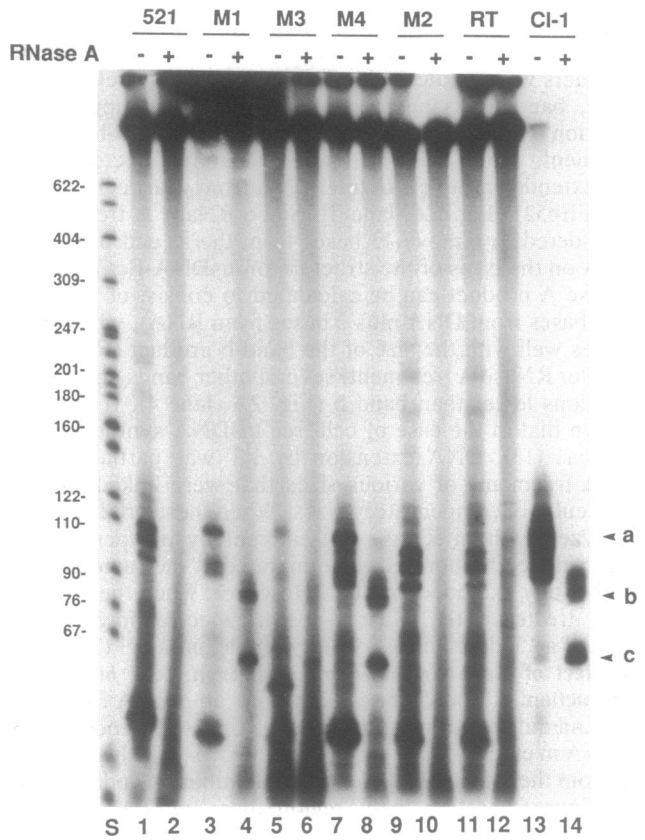

FIG. 3. msDNA production in *S. cerevisiae* harboring YEp521-M1, -M3, and -M4. msDNA production was analyzed by the AMV RT extension method as described in Fig. 2. The RT reaction mixture was analyzed in a 4% acrylamide/8 M urea gel with and without RNase A treatment. Lane S, pBR322 digested with *Msp* I and ^{32}P-labeled with Klenow fragment (molecular size standards; numbers at left indicate sizes of fragment in bases); lanes 1 and 2, AMV RT extension products with total RNA from yeast cells harboring YEp521; lanes 3 and 4, YEp521-M1; lanes 5 and 6, YEp521-M3; lanes 7 and 8, YEp521-M4; lanes 9 and 10, YEp521-M2; lanes 11 and 12, YEp521-RT; lanes 13 and 14, *E. coli* strain Cl-1.

Most important, neither band b nor band c was detected in the control cells harboring YEp521 (Fig. 3, lane 2), YEp521-M2 (lane 10), or YEp521-RT (lane 12). These results indicate that both RT and the *msr–msd* region are required for msDNA production. Note that in the control experiments without RNase A treatment (lanes 1, 9, and 11) there was a band migrating at a similar position as band a, whereas neither band b nor band c appeared after RNase A treatment. In the YEp521-M3 construct, msDNA production was somewhat reduced (lanes 5 and 6); the reason for this reduction is not known. However, in YEp521-M4, a substantial increase of the production of msDNA (lanes 7 and 8) was observed. This increase in msDNA production is likely to be due, at least in part, to the improvement of RT production by the M4 construction.

Characterization of msDNA-Ec67 Produced in Yeast. To further characterize the synthesis of msDNA in yeast cells, Southern blot analysis was performed with the total RNA fraction isolated from a 0.6-ml culture of yeast cells harboring YEp521, YEp521-M1, -M2, -M4, or -RT. The 140-bp *Hin*dIII–*Bam*HI fragment was isolated from YEp521-M2 (Fig. 1), nick-translated, and used as a probe. YEp521-M1 (Fig. 4*A*, lane 2), -M4 (lane 3), and -M2 (lane 4) produced low molecular weight bands that hybridized with the probe. The bands indicated by an arrowhead in YEp521-M1 (lane 2) and -M4 (lane 3) migrated at the same position as RNase A-treated msDNA-Ec67 produced in *E. coli* harboring pCl-1EP5a (lane 6). These bands were RNase A-resistant (Fig. 4*B*, lanes 2 and 3). The bands that migrated a little slower than msDNA-Ec67 in YEp521-M1 (lane 2), -M4 (lane 3), and -M2 (lane 4) are likely to be primary RNA transcripts, since they disappeared after RNase A treatment (Fig. 4*B*, lanes 2–4). These results together with the results from Figs. 2 and 3 demonstrate that msDNA-Ec67 was produced in yeast cells. The RNase A-resistant band that migrated a little slower than msDNA-Ec67 in lane 3 of Fig. 4*B* is probably a molecule such as the band-b molecule illustrated in Fig. 2*B*, but its exact structure is unknown.

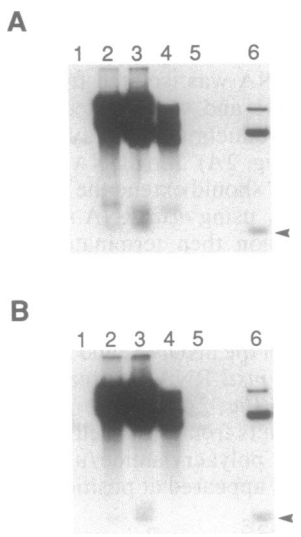

FIG. 4. Southern blot hybridization of msDNA-Ec67 produced in *S. cerevisiae*. (*A*) Total RNA fractions prepared from a 0.6-ml culture of yeast cells (1.7×10^7) harboring YEp521 (lane 1), YEp521-M1 (lane 2), -M4 (lane 3), -M2 (lane 4), or -RT (lane 5) were analyzed. msDNA-Ec67 from *E. coli* CL83, harboring pCl-1EP5a, was prepared and msDNA from 2.7×10^5 cells was applied to lane 6. After blotting to a nylon membrane filter, msDNA-Ec67 was detected with the nick-translated 140-bp *msr–msd* DNA fragment as a probe. Arrowhead indicates position of msDNA-Ec67. (*B*) Same samples applied in *A* were treated with RNase A prior to gel electrophoresis. Arrowhead indicates position of msDNA-Ec67.

Yeast endogenous RT associated with Ty elements (20) is apparently unable to produce msDNA-Ec67, since cells harboring YEp521-M2 could not produce the msDNA (Fig. 3, lanes 9 and 10, and Fig. 4*B*, lane 4).

Copy Number of msDNA-Ec67 in Yeast. The amount of msDNA produced in yeast cells was calculated for YEp521-M4 as follows; in Fig. 3, the sample applied to lane 8 was from $\approx 1.4 \times 10^7$ yeast cells, whereas the sample applied to lane 14 was from $\approx 7.8 \times 10^6$ *E. coli* Cl-1 cells. Since the density of bands b plus c in lane 14 is approximately twice that in lane 8, and msDNA-Ec67 exists at about 500 molecules per cell (9), the copy number of msDNA-Ec67 for YEp521-M4 was estimated to be about a hundred molecules per cell. A similar estimate was obtained by comparing the intensities of msDNA bands in lanes 3 and 6 in Fig. 4. Note that *E. coli* cells harboring pCl-1EP5a (Fig. 4, lane 6) produce $\approx$100 times more msDNA-Ec67 than *E. coli* Cl-1 (M.-Y. Hsu, S.I., and M.I., unpublished results).

DISCUSSION

The results demonstrate that retron Ec67 under the *GAL10* promoter produces a unique DNA product in yeast cells. This product is identified as msDNA-Ec67 from the following facts. (*i*) As expected from the branched RNA-linked structure of msDNA-Ec67, the DNA strand could be extended from its 3′ end by AMV RT, yielding a compound of 118

nucleotides. (*ii*) RNase A treatment of this compound shortened it by 13 nucleotides. The RNase A-resistant structure consisted of 105 nucleotides, which agrees well with the size of the RT-extended product of msDNA-Ec67 treated with RNase A (67 nucleotides from the original single-stranded DNA plus 36 nucleotides added by RT extension at the 3′ end plus 2 nucleotides from *msd* RNA left attached at the 5′ end of the DNA strand after RNase A treatment). (*iii*) When analyzed by Southern blot hybridization, the product hybridized with the *msr–msd* DNA fragment and migrated at the same position as msDNA-Ec67 produced in *E. coli*. (*iv*) Its production was dependent upon the RT gene in the retron.

The msDNA production in yeast was improved by moving the RT gene in front of the *msr–msd* region from its original position in the retron (downstream of the *msr–msd* region). This improvement was probably due to increased production of RT as a result of elimination of 12 AUG codons between the transcriptional initiation site of the *GAL10* promoter and the initiation codon of the RT gene. The amount of msDNA thus produced was estimated to be about a hundred molecules per cell. Although its cellular localization remains to be determined, the bacterial retron may be a novel system to produce stable single-stranded DNAs as well as RNAs in eukaryotic cells. In particular, the *in vivo* production of a stable single-stranded DNA by means of cDNA synthesis may be artificially manipulated so that a single-stranded DNA of a desired sequence can be produced. It may then be used as an antisense DNA or for the formation of a triple helix to block the expression of a specific gene (21). Antisense DNA could be more effective in some cases than antisense RNA for blocking mRNA translation, since RNA targets hybridized with antisense DNA become susceptible to RNase H digestion. In a preliminary experiment, an *Xho* I site was introduced in the *msd* sequence of YEp521-M4 and a foreign DNA fragment of 50 bp was inserted in the *Xho* I site. msDNA was detected from yeast cells transformed with the newly constructed plasmid.

It is most likely that msDNA in yeast cells is produced by the same mechanism as in *E. coli* cells, since in both cases two genetic elements, the *msr–msd* region and the RT gene, are required for msDNA production. The *msr–msd* region can be functional whether it is located upstream or downstream of the RT gene. This is consistent with the results in *E. coli*, where both elements can function in trans under separate promoters (9).

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