5262–5270 *Nucleic Acids Research, 2005, Vol. 33, No. 16*
doi:10.1093/nar/gki819

Localization, mobility and fidelity of retrotransposed Group II introns in rRNA genes

Lori H. Conlan, Matthew J. Stanger, Kenji Ichiyanagi and Marlene Belfort*

Wadsworth Center, Center for Medical Science, New York State Department of Health,
150 New Scotland Avenue, Albany, NY 12208, USA

Received July 18, 2005; Revised and Accepted August 19, 2005

ABSTRACT

We previously showed that the group II *Lactococcus lactis* Ll.LtrB intron could retrotranspose into ectopic locations on the genome of its native host. Two integration events, which had been mapped to unique sequences, were localized in the present study to separate copies of the six *L.lactis* 23S rRNA genes, within operon B or D. Although further movement within the bacterial chromosome was undetectable, the retrotransposed introns were able to re-integrate into their original homing site provided on a plasmid. This finding indicates not only that retrotransposed group II introns retain mobility properties, but also that movement occurs back into sequence that is heterologous to the sequence of the chromosomal location. Sequence analysis of the retrotransposed introns and the secondary mobility events back to the homing site showed that the introns retain sequence integrity. These results are illuminating, since the reverse transcriptase (RT) of the intron-encoded protein, LtrA, has no known proofreading function, yet the mobility events have a low error rate. Enzymatic digests were used to monitor sequence changes from the wild-type intron. The results indicate that retromobility events have $\sim 10^{-5}$ misincorporations per nucleotide inserted. In contrast to the high RT error rates for retroviruses that must escape host defenses, the infrequent mutations of group II introns would ensure intron spread through retention of sequences essential for mobility.

INTRODUCTION

Group II introns are mobile retroelements that insert site-specifically into unoccupied sites of intronless alleles [reviewed in refs (1,2)]. They are found in bacteria, archaea and the organelles of eukaryotes (2,3). Both splicing and mobility pathways have been described for group II introns in yeast and bacteria (1,2).

Introns retrohome into a specific DNA target, known as the homing site, and retrotranspose to ectopic sites that resemble this homing site (1,2). Intron mobility is catalyzed by a ribonucleoprotein (RNP) complex containing the excised intron RNA and the intron-encoded protein (IEP). The IEP has RNA maturase, reverse transcriptase (RT) and in some cases DNA endonuclease activities. In retrohoming, the RNP complex recognizes the exon junction of a specific DNA target. The maturase activity facilitates reverse splicing of the lariat RNA into the top strand of the DNA target. For the *Lactococcus lactis* LtrB intron (Ll.LtrB), the endonuclease of the IEP cleaves the bottom strand 9 bp 3′ to the intron insertion point. The free 3′-hydroxyl end is then used as a primer for the IEP RT to generate a new cDNA strand templated by the intron RNA. Finally, the reaction is completed by host repair functions (4).

We have previously shown that the Ll.LtrB group II intron can invade ectopic chromosomal sites within the *L.lactis* genome (5,6). These invasions were mapped to *ca*. sixty locations within the chromosome, including six integrations into the 23S rRNA genes. Events #4 and #7, which retrotransposed into different locations of the 23S rRNA gene, are analyzed here since they exhibited the highest splicing activity among those tested (5). These introns, which are marked by a kan^R gene used to detect their original chromosomal integration, provide us with a system by which to study intron proliferation within bacteria.

Here we show that each of the two chosen introns invaded a different copy of the six 23S rRNA genes. The point of invasion is displayed on the surface of the ribosome, as modeled on the structure of the *Haloarcula marismortui* ribosome (7,8). Multiple chromosomal intron invasions are not seen, leading us to hypothesize that increased numbers of intron invasions would compromise the fitness of that cell as compared to the wild-type. We show that each retrotransposed group II intron

*To whom correspondence should be addressed. Tel: +518 473 3345; Fax: +518 474 3181; Email: belfort@wadsworth.org
Present address:
Kenji Ichiyanagi, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan

is capable of re-integrating precisely into the LtrB homing site. These results are the first indication that a retrotransposed intron retains mobility properties. The remobilized introns preserve their sequence, suggesting that the mobility reaction has high fidelity. The faithfulness of the remobilized introns is compared with that of the other retro-elements and with that of retroviruses.

MATERIALS AND METHODS

Bacterial strains and growth conditions

L.lactis strains were cultured in M17 medium (Difco) containing 0.5% glucose, at 30°C without aeration. Spectinomycin (Spc) (300 μg/ml) and/or kanamycin (Kan) (20 μg/ml) was added to the medium as needed. *Escherichia coli* strains were cultured in Luria Broth at 37°C. For *E.coli* cultures, Spc and/or Kan was added at 50 μg/ml as needed.

Mobility assays

The retrohoming recipient, pMN1343, contains a 211 bp homing site on a HindIII fragment in the Spc^R shuttle vector pDL278 (9,10). Plasmid pMN1343-rev has the homing site in the opposite orientation to pMN1343. It was made by cutting pMN1343 with HindIII, then re-ligating, and sequencing for a change in the direction of the homing site.

Plasmid recipients were transformed into *L.lactis* by electroporation. DNA was isolated from at least 10 *L.lactis* colonies, by incubation in 30 mg/ml lysozyme for at least 30 min at 37°C, followed by the standard alkaline lysis miniprep (11). This DNA was transformed into electrocompetent *E.coli* DH5α cells, which were plated on medium containing Spc and Kan. If the plasmid recipient inherited the intron, it would also carry the kan^R gene, thereby allowing it to survive this second selection. Plasmid DNA was isolated by Qiagen miniprep and sequenced.

Genomic DNA preparation and pulsed-field gel electrophoresis (PFGE)

L.lactis cell plugs for PFGE were prepared as described previously (12). Briefly, cells were grown overnight, and then mixed 1:1 with 2% low-melting point agarose. The cell plugs were treated with 5 mg/ml lysozyme, then with 1 mg/ml proteinase K and finally with 10 U of I-CeuI, where each incubation was at 37°C for at least 30 min. The plugs were buffer-exchanged with 10 mM Tris HCl (pH 7.5), and 100 mM EDTA and stored at 4°C until use.

PFGE-grade agarose gels (1%) (Bio-Rad) were made up in 1× TBE. PFGE was performed in a CHEF system (Bio-Rad) with the tank buffer being 0.5× TBE. Gel running conditions were standard for separating phage λ DNA (Bio-Rad). Gels were run at 200 V, with switch times of 50–90 s for 22 h at 12°C.

Genomic DNA preparation for agarose gel electrophoresis

Genomic DNA from *L.lactis* cells containing a chromosomal intron (6) was isolated by lysis in 25% sucrose, 30 mg/ml lysozyme for 15 min at 37°C, followed by 40 mM Tris-acetate (pH 7.8), 20 mM NaOAc, 1 mM EDTA, 1% SDS and 0.1 mg/ml RNaseA for 30 min at 37°C (13). NaCl was added to a final concentration of 1.2 M; the DNA was then extracted with phenol–choloroform and ethanol-precipitated. Genomic DNA (10 μg) was digested with 1 U of EcoRI overnight at 37°C, and then extracted with phenol–choloroform and ethanol-precipitated. Cleavage products were separated on a 0.6% agarose gel in Tris-acetate-EDTA.

Southern hybridization

DNA probes for southern blots were synthesized using the PCR with *Taq* DNA polymerase. Oligonucleotide sequences are listed in Supplementary Data. For the 23S rRNA gene, 450 bp of NZ9800ΔLtrB genomic DNA were amplified with primers W1947 and W1948. For the LtrB probe, 560 bp of intron DNA were amplified with primers W635 and W653. PCR products were separated on a 1% agarose gel. The correct fragment was isolated from the agarose gel and purified using the GeneClean kit (Bio101). The fragment was labeled with [α^{32}P]dCTP using the Random Prime Labeling Kit (Invitrogen). Unincorporated nucleotides were removed using a Sephadex G50 spin kit (GE LifeSciences), and the purified probe was boiled before use.

Agarose gels or PFGE gels were treated with 0.25 M HCl for 30 min, rinsed with distilled water, and treated with 0.4 M NaOH, 1.5 M NaCl for a further 30 min. The DNA was transferred to an H-bond+ membrane using the VacuGene Pump system (GE LifeSciences) for 45 min in 0.4 M NaOH. The membrane was rinsed with 15 mM NaCl, 1.5 mM sodium citrate (2× SSC) (pH 7) to neutralize the base. Then, 7 ml of RapidHyb solution (GE LifeSciences) was added to the membrane, which was allowed to incubate for 30 min at 42°C. The appropriate radiolabeled probe was added to the solution and allowed to incubate for another 2 h at 42°C. The membrane was then washed with 100 ml of 6× SSC at 65°C for 15 min and 2× SSC for another 15 min at 65°C. The membrane was dried, wrapped in SaranWrap and set to a phosphoimager screen overnight. The screen was scanned and data were analyzed using ImageQuant software (Molecular Dynamics).

SURVEYOR endonuclease cleavage

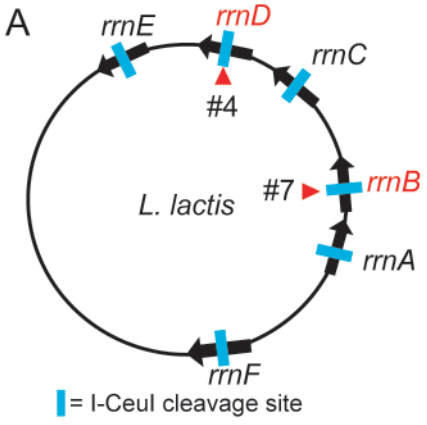
The intron was amplified by the PCR using nested primer sets to give ~1000 bp products. The products were mixed 1:1 with a wild-type sample and annealed following published protocols (14). These samples were digested with 1 μl of SURVEYOR nuclease (Transgenomic) and 1 μl of SURVEYOR enhancer in the PCR buffer. Cleavage reactions were performed at 42°C for at least 30 min. The reaction was quenched by the addition of stop solution supplied by the manufacturer. Products were separated by agarose gel electrophoresis and visualized after ethidium bromide staining. Possible mutations were sequenced.

RESULTS

Localization of the group II intron within the chromosomal 23S rRNA genes

The sequence context of two retrotransposed introns that splice efficiently (events #4 and #7) indicated that they were both in a 23S rRNA gene (*rrnl*) (5). Initially, we wished

to determine whether these chromosomal invasions were located within the same or different ribosomal operons, and whether they spread among the operons. The *L.lactis* chromosome contains six copies of the 23S rRNA gene within operons A–F (Figure 1) (12). A southern blot was performed with DNA from *L.lactis* cells carrying the retrotransposed introns after digestion with I-CeuI and separation by PFGE. I-CeuI cleaves the *L.lactis* genome six times, once in each ribosomal RNA operon, to generate six fragments ranging in size from 22 to 1533 kb (Figure 1, 23S probe). The control *L.lactis* strain NZ9800, containing a chromosomal intron (15), shows a band of 1533 kb when probed for the intron. The intronless strain NZ9800ΔLtrB was generated from NZ9800, and was the host in which events #4 and #7 were isolated (5). Whereas there was no signal with the intron probe for DNA from strain NZ9800ΔLtrB, DNA from cells containing event #4 and from cells containing event #7 showed bands of 240 and 80 kb, respectively. These results indicate that each intron targeted a separate rRNA operon. Analysis of the intron invasion sequence in reference to the position of the I-CeuI recognition site determined that the intron targeted the 23S rRNA gene in operon D for event #4 and that in operon B for event #7 (Figure 1).



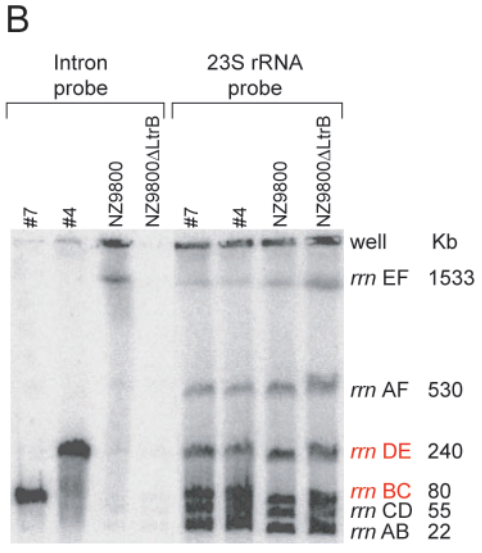


Figure 1. Mapping of intron insertions on the *L.lactis* chromosome. (**A**) An *rrn* operon map of the *L.lactis* chromosome. The six rRNA operons are located throughout the chromosome, as symbolized by black arrows. The I-CeuI digestion sites are indicated by blue boxes, and intron invasion points by red arrowheads. (**B**) Introns on the *L.lactis* chromosome are visualized by southern hybridization. *L.lactis* genomic DNA was digested with I-CeuI and separated by PFGE. The DNA was probed by two oligonucleotides, one specific to the intron and the other to 23S rRNA sequence as described in Materials and Methods. Bands into which intron invasion had occurred are indicated in red. There appears to be a gradient in the efficiency of DNA transfer from low to high molecular-weight bands.

Probing of intron stability in the chromosome

To examine the stability of the intron in the chromosome, we monitored possible intron movement within the chromosome as a function of generation number. *L.lactis* cells were passaged for 1000 generations by diluting the culture, and allowing growth to late log phase before the next passage. Genomic DNA from every 100th generation was digested by I-CeuI and examined using southern blot analysis, to determine whether the intron had moved. This was done with and without antibiotic selection pressure, to determine whether selection might influence intron mobility and maintenance. Using both the PFGE assay and a more sensitive nested-PCR assay, no intron spread or loss was detected (data not shown). These results are noteworthy, since multiple *rrnl* genes provide many chromosomal locations that are possible targets of the intron.

Four possibilities could account for our failure to observe further intron movement. The first is that intron remobilization is extremely rare, and below the detection limit of our assay. The second possibility is that a retrotransposed intron imposes an immunity to further movement, as phage Mu does (16,17). The third possibility is that the introns have lost their mobile properties, as often is the case with retrotransposed LINE elements (18). Finally, the presence of two introns in the same cell might present a growth disadvantage, such that these cells would be selected against in culture. Whereas we are not able to assess the rarity of re-mobilization events due to the detection limits of the assay, the other three possibilities are considered in the sections below and in the Discussion.

Testing whether group II introns impose immunity

To address the immunity hypothesis, we examined retrotransposition into a *L.lactis* strain that contains a resident Ll.LtrB intron (15). Intron retrotransposition events had been selected in NZ9800 cells harboring the wild-type Ll.LtrB intron on the chromosome (6). To probe for the existence of multiple introns, genomic DNA from these cells was digested with EcoRI, which does not cleave within the intron. A representative sample of 20 events, shown in Figure 2, was probed for the original chromosomal intron copy and the newly retrotransposed intron. The intron probe should highlight one band for the wild-type chromosomal intron and one or more bands for additional intron invasion events. The southern blot reveals that, besides the native copy of the Ll.LtrB intron, there is one additional band of differing size in each of the events (Figure 2, lanes 5–8). As expected, only one band was observed for events #4 and #7, since the host did not have a resident group II intron. The observation that chromosomes bearing a resident copy of the intron can obtain an additional

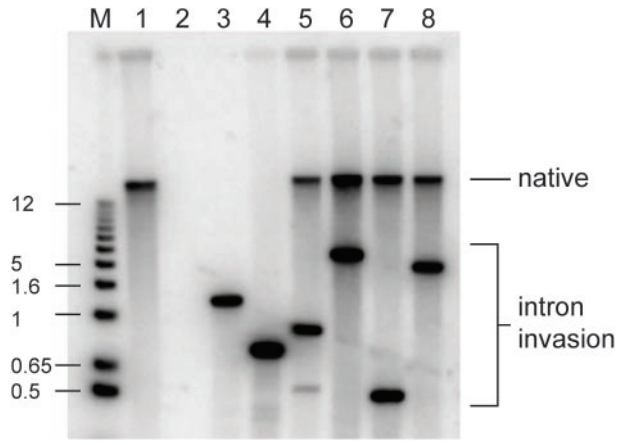


Figure 2. Secondary intron invasions into the *L.lactis* chromosome. Genomic DNA was digested with EcoRI, separated by agarose gel electrophoresis, and hybridized with a probe to the intron. Lanes: M, 1 Kb+ markers (Invitrogen); 1, NZ9800 (probed for resident intron); 2, NZ9800ΔLtrB; 3,4 NZ9800ΔLtrB event #4 and event #7, respectively; 5–8, representative examples of 20 tested events, highlighting the resident intron and in each case an additional intron invasion. The faint band in lane 5 is artifactual.

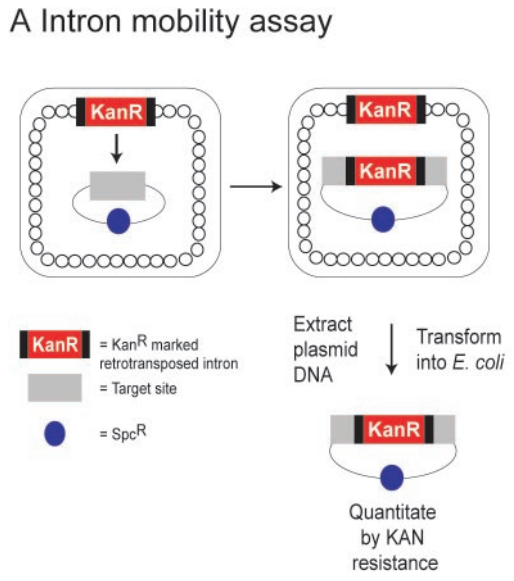
intron argues against intron-imposed immunity. This point is particularly relevant given that the native intron in NZ9800 is active in mobility (data not shown).

Determining whether retrotransposed group II introns retain mobility

To test whether the retrotransposed chromosomal introns retain mobility properties, we probed their integration into a plasmid containing the LtrB homing site embedded in a 211 bp HindIII fragment on plasmid pMN1343. Successful intron movement from the *L.lactis* chromosome to the plasmid would render *E.coli* cells transformed by these plasmids Spc^R and Kan^R (Figure 3A). Using this assay system, the targeting efficiency, a measure of the number of intron-containing plasmids per recipient, was 5.9% for event #4 and 2.2% for event #7 (Figure 3B). These targeting efficiencies were two orders of magnitude greater than those for the plasmid without the homing site, indicating that the intron efficiently targeted the homing site, despite the absence of any homology between flanking DNA on the donor chromosome and the intron homing site on the plasmid. This indicated that the retrotransposed introns had maintained activity. These results are in accord with the observation that the retrotransposed introns were of wild-type sequence (see below). Consistent with the retrohoming pathway, mobility was also high when the orientation of the homing site was switched in the plasmid, although a 1.5- to 2-fold drop was observed for both event #4 (3.4%) and event #7 (1.1%) relative to the target in the opposite orientation. Retrohoming pathways, using dsDNA targets, invade the homing site whether in the sense or anti-sense direction, or the leading or lagging strand of DNA synthesis (19). If there were a strong strand bias, this would have suggested that the intron was using a ssDNA pathway, more characteristic of retrotransposition (6).

Location of the introns on the structure of the ribosome

We modeled the intron invasion points on the structure of the ribosome, assuming that the ribosomal structure of *L.lactis* is



B Intron mobility to a plasmid recipient

	Mobility			
Recipient	Donor #4	(n)	Donor #7	(n)
O	<0.03 ± 0.001	(7)	<0.02 ± 0.01	(8)
HS+	5.9 ± 1.4	(8)	2.2 ± 0.9	(8)
HS-	3.4 ± 0.7	(8)	1.1 ± 0.7	(9)

Figure 3. Assay to monitor intron movement from chromosome to plasmid. (**A**) Intron mobility assay in *L.lactis*. Intron movement from the chromosome to the Spc^R-plasmid, pMN1343, was scored after re-transformation of plasmids into *E.coli*. (**B**) Quantitation of intron movement from the chromosome to the plasmid homing site. Percent mobility is scored as the percentage of Spc^RKan^R/Spc^R colonies, with n representing the number of independent trials. Recipients: O, plasmid pDL278 with no homing site; HS+, plasmid pMN1343 with homing site in the original (+) orientation in pDL278; HS−, plasmid pMN1343 with homing site in the reverse (−) orientation in pDL278.

similar to that of the archaeon *H.marismortui*, which has been crystallized (7,8). Based on the predicted secondary structure of the 23S rRNA of *L.lactis*, both events are in stem–loop structures of the RNA, event #4 in domain II and event #7 in domain I (Figure 4A and B) (20). These are both surface locations (Figure 4C and D). The corresponding stem–loop in *H.marismortui* for event #4 is slightly occluded by ribosomal protein L32E. However, L32E is present in archaea but absent in bacteria (21), thereby providing a surface location for event #4 in our bacterial system. These two events are positioned on the 50S ribosomal particle away from the surface of interaction with the small subunit. These invasion points on the surface of the ribosome may present an optimal location for function of both the intron and the ribosome.

Fidelity of retromobility

Initial sequencing of the retrotransposed chromosomal introns (events #4 and #7) revealed absolute sequence conservation relative to the parental intron. Kinetic experiments for direct comparison of enzyme fidelity between retroviral and group II

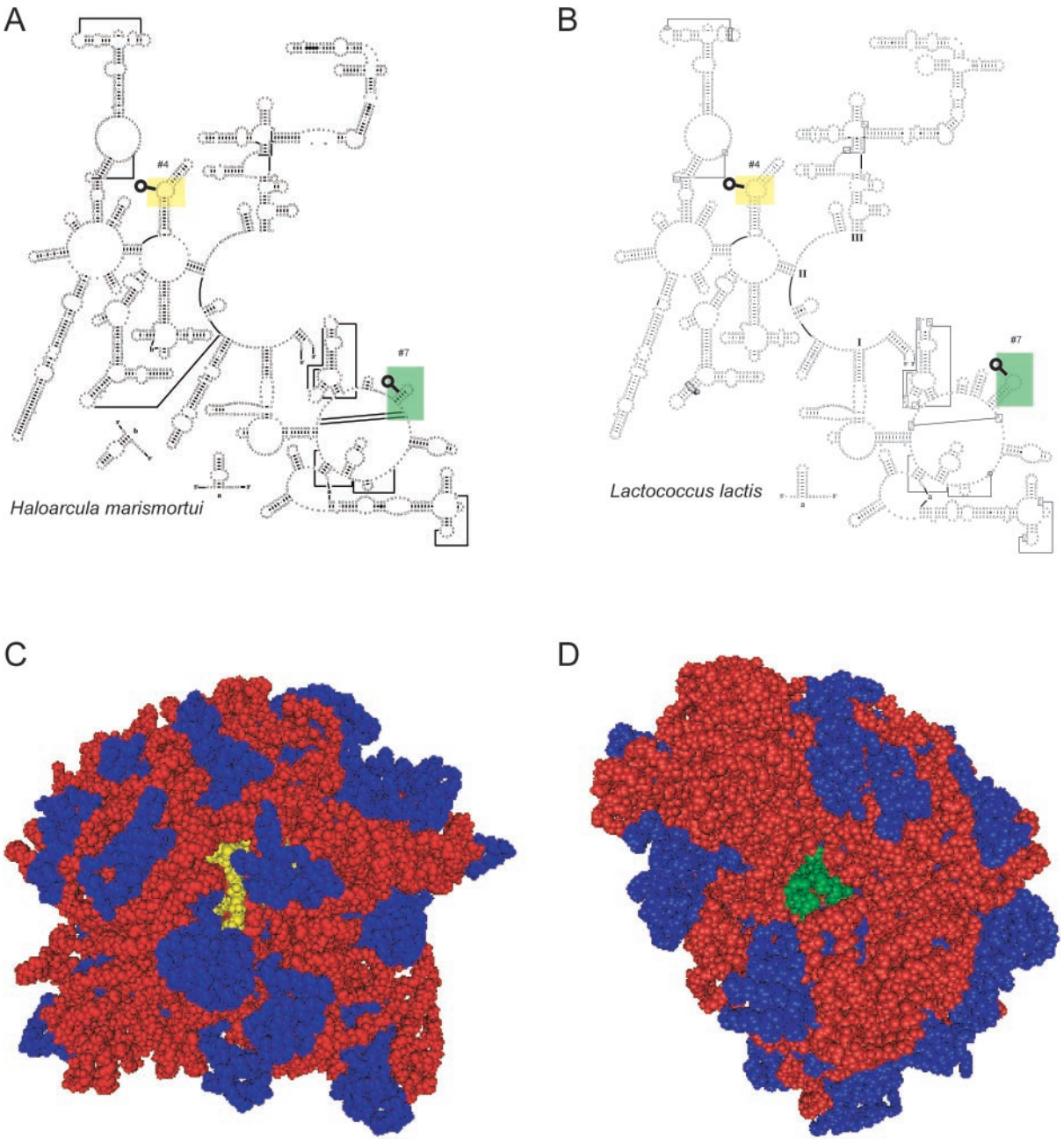


Figure 4. Modeling of the location of the intron on the ribosome. A and B. Secondary structures of the 5′ half of the 23S rRNA of *H.marismortui* (**A**) and *L.lactis* (**B**) The position of the introns in domains I and II on the secondary structures are shown in yellow for event #4 and in green for event #7. C and D. Three-dimensional structure of the 50S *H.marismortui* ribosomal subunit (8) in two different views. The 23S rRNA is in red and proteins are in blue. The location of the intron in event #4 is in yellow (**C**), and in event #7 is in green (**D**). Ten nucleotides to each side of the invasion site are colored. Protein L32, not present in bacteria, has been removed. Modeling was done using Cn3D software from NCBI.

intron RTs were precluded because the activity of LtrA was not amenable to assay under Michaelis–Menton conditions. Kinetic interpretation was further hindered by the fact that neither the amount of active enzyme nor the on and off rates of each enzymatic step were known. Therefore, we tested the fidelity of retromobility *in vivo*. We adapted technology designed for single nucleotide polymorphism detection, using a mismatch-specific DNA endonuclease to distinguish heteroduplexes formed between mutant and wild-type sequences (Figure 5A–D) (22). The SURVEYOR endonuclease cleaves both strands of heteroduplex DNA 3′ to the mutation. The positive controls for our assay were intron branch-point A mutations, ΔA or A→C, which were compared to the wild-type intron. After enzymatic cleavage, the heteroduplexes yielded digestion products for both the ΔA and A→C mutations, but the wild-type homoduplex remained uncleaved (Figure 5B). Genomic DNA from 30 different retrotransposition events in *L.lactis* was amplified with four primer sets (Figure 5A). The control intron for these reactions contained the kan^R gene but no group I intron, paralleling the

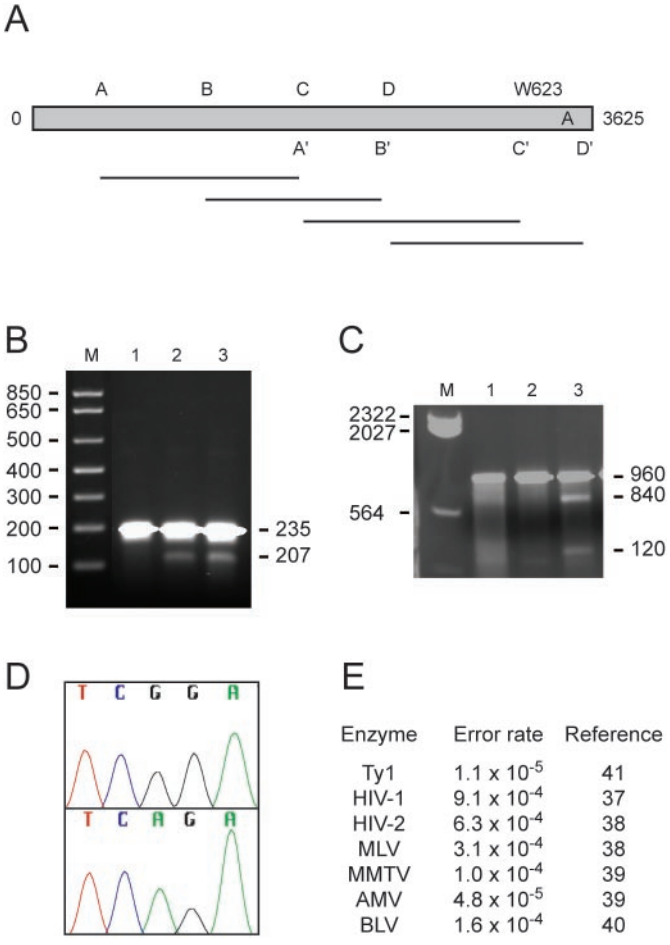


Figure 5. Fidelity of the Ll.LtrB mobility reaction. (**A**) Experimental design of PCR and sequencing primers. The intron is shown as a grey bar, with the bulged A, which was mutated in control reactions, noted. Oligonucleotide sequences, denoted A through D and A′ through D′, can be found in the Supplementary Data. Horizontal lines between primers denote PCR products. (**B**) Control reactions. Wild-type intron DNA, or that with a ΔA or A→C mutation at the branchpoint, was amplified with primers W596 and W623. Primer W596 anneals to the plasmid downstream of the 5′ splice site and is not represented in panel A. Primer W623 is the 5′ primer annealing at nucleotide 3405. Samples were duplexed with wild-type DNA, cleaved with SURVEYOR enzyme and separated on an agarose gel stained with ethidium bromide. Lane M, 1 Kb+ markers; lane 1, wild-type, homoduplex; lane 2, ΔA heteroduplex; lane 3, A→C heteroduplex. (**C**) Endonuclease screen of retrotransposition events. DNA was PCR amplified with primers IDT0205 and IDT0118, duplexed with wild-type DNA and digested as described in Materials and Methods. The stained agarose gel shows PCR products subjected to the endonuclease. Lane M, size markers in bp; lane 1, wild-type PCR fragment; lane 2, wild-type DNA duplexed with experimental DNA bearing no mutations; lane 3, wild-type duplexed with experimental DNA bearing a mutation. (**D**) Chromatogram from sequencing of PCR products of wild-type DNA (top panel) and possible mutations (C, lane 3) (bottom panel). Sequencing was done with primer IDT0208. (**E**) Comparison of various RT error rates. The error rate is the number of C misincorporations opposite an A template.

sequence of retrotransposition events, where the group I intron would have spliced out. The experimental and control PCR products were heteroduplexed and digested. Only one retrotransposition event tested showed a digestion pattern after cleavage with the endonuclease (Figure 5C; lane 3). This mutation is not due to a PCR polymerase error, since multiple independent reactions showed this mutation. This mutation was sequenced and determined to be a G to A transition 167 bp upstream of the 3′ splice site (Figure 5D). These results suggested an error rate for the homing reaction of $\sim 10^{-5}$ misincorporations per nucleotide inserted.

DISCUSSION

Localization and maintenance of retrotransposed introns in different rRNA operons

This work characterizes retrotransposed introns in the *L.lactis* chromosome. The group II Ll.LtrB intron, which had been localized previously to two separate sites within the 23S rRNA gene (5), was shown here to have targeted different copies of the six available 23S rRNA genes, within the B and D operons. We have used these events to answer questions about intron maintenance and the fidelity of the retromobility process.

Despite the proven activity of the two retrotransposed rRNA introns (Figure 3), and the demonstration that resident group II introns do not confer immunity to subsequent invasions (Figure 2), movement to secondary chromosomal sites, by either transposition to ectopic sites or recombination within rRNA operons, could not be demonstrated. It is unclear whether these observations are attributable to the inefficiency of retrotransposition and/or the instability of invasive elements inherited into rRNA genes. Due to the existence of large regions of sequence similarity between rRNA genes, recombination between these operons can be fairly high, with a rate of 10^{-3} to 10^{-9} events per cell division (23,24). However, maintenance of rRNA recombination products is rare, likely due to a decrease in fitness of the organism, implying that introns spread via recombination would rarely become permanently fixed in the genome. This notion is supported by evidence that five of the seven *E.coli* rRNA operons were necessary for near-optimal growth on complex medium (25). Therefore, we surmise that wild-type cells would outgrow those with multiple intron invasions within the rRNA operons, thereby preventing recolonized introns within a population from becoming stably established.

Location and maintenance of the group II intron could be based on the position on the tertiary structure of the 23S rRNA, such that presence of the intron would be consistent with ribosome function. The two insertion events analyzed in this study indeed suggest that the Ll.LtrB intron is maintained on the surface of the ribosome. Although natural group I and group II introns within the 23S rRNA gene are not always solvent accessible (7), it is not known if these natural introns remain capable of splicing. These natural events are therefore not inconsistent with the hypothesis that the invasions analyzed in this study are maintained on the surface of the ribosome to preserve its function. This position would provide the intron with an acceptable location for splicing, and also maintain the overall assembly of the ribosome for those circumstances in which splicing is incomplete. This hypothesis is also consistent with a construct in which the intron has been cloned into the surface loop 98 of the 23S rRNA gene. Not only is the group II intron able to splice from loop 98, but the rRNA-group II intron RNA chimera can be packaged to form a stable ribosome (Slagter-Jäger, J., Allen, G., Smith, D., Frank, J. and Belfort, M., unpublished data). Thus, the structural

organization of the 23S rRNA in the ribosome may dictate tolerance to an intron integration event.

Retrotransposed introns retain mobile properties

Both model events, #4 and #7, were transcribed from the chromosome and retained splicing capabilities (5). Here, we built upon that knowledge to show that these introns retain mobility properties as well. The observed movement of the intron from either of the two sites on the chromosome to the plasmid homing site, which bears no sequence identity with the sequences flanking the intron in the 23S rRNA gene, is consistent with the model for retrohoming, in that the retrohoming process occurs in the absence of homologous recombination (9,19). Additionally, in the experiment shown in Figure 3B, there was a degree of orientation dependence for both events #4 and #7, with retrohoming occurring at about one-half of the frequency when the homing site was inverted relative to the origin. This suggests that in addition to the standard retrohoming pathway, a replication-dependent pathway may be accounting for some fraction of events. Perhaps the rolling-circle replication of the plasmid provides single-stranded DNA targets. Single-strand DNA invasion is preferred for *L.lactis* retrotransposition (6,26), and also accounts for some such events in *E.coli* (27), possibly providing an alternative mobility pathway for these secondary events to invade the inverted homing site.

LINE elements, which are also non-LTR retrotransposons that encode proteins with endonuclease, RNA-binding and RT functions, have been shown previously in both human and mouse to retain secondary retrotransposition abilities (18,28). However, the majority of LINEs become immobile after the first retrotransposition event, due to 5′-truncations. Indeed, all but 30–60 of ~100 000 LINEs in the human genome are 'dead on arrival' (18). This situation contrasts dramatically with group II introns. Not only are the two events studied here intact and active, but the vast majority of retrotransposed group II introns in a variety of bacteria and yeast represent complete copies of the element (6,27,29,30). This is also true for Ty1 elements in yeast (31). Therefore, although group II introns closely resemble LINE elements, they have evolved mobility mechanisms that maintain function, to perpetuate their spread.

Integrity of mobilized introns suggests fidelity of the intron-encoded RT

The activity of the retrotransposed introns and the fidelity of observed retrotransposition events prompted a systematic analysis of the error frequency of retromobility. Rigorous fidelity assays typically involve detailed kinetic assays (32). The relative inactivity of LtrA and the LtrA-RNP complex (33) precluded *in vitro* RT fidelity measurements. Therefore, we analyzed *in vivo* mobility events from 30 retrotransposition events in *L.lactis*, representing 100 000 bases of intron sequence. Traditional *in vivo* measurements of reverse transcription fidelity involve inactivation of the nonessential α-complementation activity of the *lacZ* gene in bacteriophage M12mp2 (34), or DNA sequencing (35). These assays tend to be laborious and time consuming. Therefore, we adapted a screen using an enzymatic digest to detect mutations, similar to that used in SNP analysis. This approach allowed us not only to assess the error-rate associated with group II intron retromobility, but also to potentially evaluate the error profile. However, this method does not measure the fidelity of the RT directly, but rather that of the entire mobility reaction, which may reflect contributions by host cell functions (4).

Like retroviral RTs, with their relatively high error frequencies, LtrA RT lacks a 3′–5′ exonucleolytic proofreading function. The error rate of DNA polymerization by the bacterial polIII holoenzyme, with a proofreading domain, is 10^{-6}–10^{-7} per nucleotide inserted (36). In contrast, HIV-1 RT is the most error-prone RT known, with 10^{-3} and 10^{-4} misincorporations per nucleotide added (Figure 5D) [(37,38) and refs therein]. Many other retroviral RTs, including murine leukemia viruses, mouse mammary tumor virus, and avian myeloblastosis virus, lack a proofreading domain yet exhibit a fidelity at least 10-fold greater than that of HIV-1 RT (Figure 5E) (38–40). The mutation rate of the yeast retrotransposon Ty1, the only retrotransposon RT that has been examined to date, is 10^{-5} misincorporations per nucleotide added (41,42), similar to the 10^{-5} misincorporations per nucleotide shown here for LtrA. Barring recruitment of some cellular repair mechanism or inherent selection pressure within our assay, it would appear that LtrA is among the most faithful RTs known to date. The apparent fidelity of LtrA stands in sharp contrast to the behavior of *Bordetella* phage RT. This phage RT is a diversity generating system, which allows the phage to switch tropism (43), indicating that faithfulness of the copying mechanism is an adaptation to the life-style of the element.

How LtrA and the other more faithful RTs achieve accurate DNA polymerization in the absence of proofreading is not known. One possibility is that the rigidity of the dNTP-binding pocket offers less flexibility for the insertion of incorrect nucleotides (36,44–46). The primer-grip of HIV-1 RT is known to influence the G to A hypermutation (47), the most frequent mutation found for many retroviral RTs (48). Perhaps fortuitously, the only mutation detected in the Ll.LtrB mobility reaction in our study was a G to A substitution. LtrA, when modeled on the HIV-1 RT structure, has a more extended RNA-binding surface than does HIV-1 RT (49). Many protein residues that provide surface nucleic-acid interactions are also highly constrained, indicating their functional importance (50). We speculate that an increased interaction with the intron RNA, beyond just the template-primer binding track, could provide a proofreading mechanism, by allowing the LtrA RT to better scan the RNA template. Regardless, the high fidelity of this process ensures that group II introns retain the ability for subsequent mobility reactions, thereby facilitating their spread to new locations.

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

ACKNOWLEDGEMENTS

We thank Ana Rodriguez for participation in early experiments; Arthur Beauregard, Colin Coros, Vicky Derbyshire, David Edgell, Janice Pata and Coby Slagter-Jager for thoughtful discussions; Sean Conlan and John Dansereau for figure design; Maryellen Carl for manuscript preparation; and the Molecular Genetics Core for sequencing. This work was

supported by NIH grants GM39422 and GM44844 to MB and GM068370 to LHC. Funding to pay the Open Access publication charges for this article was provided by Wadsworth Center and NIH grant GM448444 to M.B.

Conflict of interest statement. None declared.

REFERENCES

1. Belfort,M., Derbyshire,V., Parker,M.M., Cousineau,B. and Lambowitz,A.M. (2002) Mobile Introns: pathways and proteins. In Craig,N., Craigie,R., Gellert,M. and Lambowitz,A. (eds), *Mobile DNA II*. ASM Press, Washington D.C., pp. 761–783.
2. Lambowitz,A.M. and Zimmerly,S. (2004) Mobile group II introns. *Annu. Rev. Genet*., **38**, 1–35.
3. Dai,L., Toor,N., Olson,R., Keeping,A. and Zimmerly,S. (2003) Database for mobile group II introns. *Nucleic Acids Res*., **31**, 424–426.
4. Smith,D., Zhong,J., Matsuura,M., Lambowitz,A.M. and Belfort,M. (2005) Recruitment of host functions suggests a repair pathway for late steps in group II intron retrohoming. *Gene Dev*., in press.
5. Cousineau,B., Lawrence,S., Smith,D. and Belfort,M. (2000) Retrotransposition of a bacterial group II intron. *Nature*, **404**, 1018–1021. [Erratum in: Nature 2001, 414, 84].
6. Ichiyanagi,K., Beauregard,A., Lawrence,S., Smith,D., Cousineau,B. and Belfort,M. (2002) Multiple pathways for the Ll.LtrB group II intron include reverse splicing into DNA targets. *Mol. Microbiol*., **46**, 1259–1271.
7. Jackson,S., Cannone,J., Lee,J., Gutell,R. and Woodson,S. (2002) Distribution of rRNA introns in the three-dimensional structure of the ribosome. *J. Mol. Biol*., **323**, 35–52.
8. Klein,D.J., Schmeing,T.M., Moore,P.B. and Steitz,T.A. (2001) The kink-turn: a new RNA secondary structure motif. *EMBO J*., **20**, 4214–4221.
9. Mills,D.A., Manias,D.A., McKay,L.L. and Dunny,G.M. (1997) Homing of a group II intron from *Lactococcus lactis* subsp. *lactis ML3*. *J. Bacteriol*., **179**, 6107–6111.
10. LeBlanc,D.J., Lee,L.N. and Abu-Al-Jaibat,A. (1992) Molecular, genetic, and functional analysis of the basic replicon pVA380-1, a plasmid of oral streptococcal origin. *Plasmid*, **28**, 130–145.
11. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
12. Le Bourgeois,P., Lautier,M., van den Berghe,L., Gasson,M.J. and Ritzenthaler,P. (1995) Physical and genetic map of the *Lactococcus lactis* subsp. *cremoris* MG1363 chromosome: comparison with that of *Lactococcus lactis* subsp. *lactis* IL 1403 reveals a large genomic inversion. *J. Bacteriol*., **177**, 2840–2850.
13. Leenhouts,K.J., Kok,J. and Venema,G. (1991) Lactococcal plasmid pWV01 as an integration vector for lactococci. *Appl. Environ. Microbiol*., **57**, 2562–2567.
14. Zhai,R.G., Hiesinger,P.R., Koh,T.W., Verstreken,P., Schulze,K.L., Cao,Y., Jafar-Nejad,H., Norga,K.K., Pan,H., Bayat,V. *et al*. (2003) Mapping Drosophila mutations with molecularly defined P element insertions. *Proc. Natl Acad. Sci. USA*, **100**, 10860–10865.
15. Shearman,C., Godon,J.J. and Gasson,M. (1996) Splicing of a group II intron in a functional transfer gene of *Lactococcus lactis*. *Mol. Microbiol*., **21**, 45–53.
16. Greene,E.C. and Mizuuchi,K. (2002) Target immunity during Mu DNA transposition. Transposome assembly and DNA looping enhance MuA-mediated disassembly of the MuB target complex. *Mol. Cell*, **10**, 1367–1378.
17. Manna,D. and Higgins,N.P. (1999) Phage Mu transposition immunity reflects supercoil domain structure of the chromosome. *Mol. Microbiol*., **32**, 595–606.
18. Kimberland,M.L., Divoky,V., Prchal,J., Schwahn,U., Berger,W. and Kazazian,H.H.,Jr (1999) Full-length human L1 insertions retain the capacity for high frequency retrotransposition in cultured cells. *Hum. Mol. Gen*., **8**, 1557–1560.
19. Cousineau,B., Smith,D., Lawrence-Cavanagh,S., Mueller,J.E., Yang,J., Mills,D., Manias,D., Dunny,G., Lambowitz,A.M. and Belfort,M. (1998) Retrohoming of a bacterial group II intron: mobility via complete reverse splicing, independent of homologous DNA recombination. *Cell*, **94**, 451–462.
20. Cannone,J.J., Subramanian,S., Schnare,M.N., Collett,J.R., D'Souza,L.M., Du,Y., Feng,B., Lin,N., Madabusi,L.V., Muller,K.M. *et al*. (2002) The Comparative RNA Web (CRW) Site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. *BMC Bioinformatics*, **2**[Erratum in: BMC Bioinformatics, 2002, 3: 15].
21. Yang,D., Kusser,I., Kopke,A.K., Koop,B.F. and Matheson,A.T. (1999) The structure and evolution of the ribosomal proteins encoded in the spc operon of the archaeon (Crenarchaeota) *Sulfolobus acidocaldarius*. *Mol. Phylogenet. Evol*., **12**, 177–185.
22. Qiu,P., Shandilya,H., D'Alessio,J.M., O'Connor,K., Durocher,J. and Gerard,G.F. (2004) Mutation detection using Surveyor nuclease. *Biotechniques*, **36**, 702–707.
23. Helm,R.A., Lee,A.G., Christman,H.D. and Maloy,S. (2003) Genomic rearrangements at *rrn* operons in Salmonella. *Genetics*, **165**, 951–959.
24. Hashimoto,J.G., Stevenson,B.S. and Schmidt,T.M. (2003) Rates and consequences of recombination between rRNA operons. *J. Bacteriol*., **185**, 966–972.
25. Condon,C., Liveris,D., Squires,C., Schwartz,I. and Squires,C.L. (1995) rRNA operon multiplicity in *Escherichia coli* and the physiological implications of *rrn* inactivation. *J. Bacteriol*., **177**, 4152–4156.
26. Ichiyanagi,K., Beauregard,A. and Belfort,M. (2003) A bacterial group II intron favors retrotransposition into plasmid targets. *Proc. Natl Acad. Sci. USA*, **100**, 15742–15747.
27. Coros,C.J., Landthaler,M., Piazza,C.L., Beauregard,A., Esposito,D., Perutka,J., Lambowitz,A.M. and Belfort,M. (2005) Retrotransposition strategies of the *Lactococcus lactis* Ll.LtrB group II intron are dictated by host identity and cellular environment. *Mol. Microbiol*., **56**, 509–524.
28. Naas,T.P., DeBerardinis,R.J., Moran,J.V., Ostertag,E.M., Kingsmore,S.F., Seldin,M.F., Hayashizaki,Y., Martin,S.L. and Kazazian,H.H. (1998) An actively retrotransposing, novel subfamily of mouse L1 elements. *EMBO J*., **17**, 590–597. [Erratum in: EMBO J 2001, 20, 2608].
29. Dickson,L., Huang,H.R., Liu,L., Matsuura,M., Lambowitz,A.M. and Perlman,P.S. (2001) Retrotransposition of a yeast group II intron occurs by reverse splicing directly into ectopic DNA sites. *Proc. Natl Acad. Sci. USA*, **98**, 13207–13212.
30. Muñoz,E., Villadas,P.J. and Toro,N. (2001) Ectopic transposition of a group II intron in natural bacterial populations. *Mol. Microbiol*., **41**, 645–652.
31. Curcio,M.J. and Garfinkel,D.J. (1994) Heterogeneous functional Ty1 elements are abundant in the *Saccharomyces cerevisiae* genome. *Genetics*, **136**, 1245–1259.
32. Creighton,S., Bloom,L.B. and Goodman,M.F. (1995) Gel fidelity assay measuring nucleotide misinsertion, exonucleolytic proofreading, and lesion bypass efficiencies. *Methods Enzymol*., **262**, 232–256.
33. Aizawa,Y., Xiang,Q., Lambowitz,A.M. and Pyle,A.M. (2003) The pathway for DNA recognition and RNA integration by a group II intron retrotransposon. *Mol. Cell*, **11**, 795–805.
34. Bebenek,K. and Kunkel,T.A. (1995) Analyzing fidelity of DNA polymerases. *Methods Enzymol*., **262**, 217–232.
35. Fersht,A.R. and Knill-Jones,J.W. (1983) Fidelity of replication of bacteriophage phi X174 DNA *in vitro* and *in vivo*. *J. Mol. Biol*., **165**, 633–654.
36. Bloom,L.B., Chen,X., Fygenson,D.K., Turner,J., O'Donnell,M. and Goodman,M.F. (1997) Fidelity of *Escherichia coli* DNA polymerase III holoenzyme. The effects of beta, gamma complex processivity proteins and epsilon proofreading exonuclease on nucleotide misincorporation efficiencies. *J. Biol. Chem*., **272**, 27919–27930.
37. Kim,B., Ayran,J.C., Sagar,S.G., Adman,E.T., Fuller,S.M., Tran,N.H. and Horrigan,J. (1999) New human immunodeficiency virus, type 1 reverse transcriptase (HIV-1 RT) mutants with increased fidelity of DNA synthesis. Accuracy, template binding, and processivity. *J. Biol. Chem*., **274**, 27666–27673.
38. Bakhanashvili,M. and Hizi,A. (1993) The fidelity of the reverse transcriptases of human immunodeficiency viruses and murine leukemia virus, exhibited by the mispair extension frequencies, is sequence dependent and enzyme related. *FEBS Lett*., **319**, 201–205.
39. Taube,R., Loya,S., Avidan,O., Perach,M. and Hizi,A. (1998) Reverse transcriptase of mouse mammary tumour virus: expression in bacteria,

purification and biochemical characterization. *Biochem. J.*, **329**, 579–587. [Erratum in: Biochem J 1998, 332, 1808].
40. Avidan,O. and Hizi,A. (1998) The processivity of DNA synthesis exhibited by drug-resistant variants of human immunodeficiency virus type-1 reverse transcriptase. *Nucleic Acids Res.*, **26**, 1713–1717.
41. Gabriel,A., Willems,M., Mules,E.H. and Boeke,J.D. (1996) Replication infidelity during a single cycle of Ty1 retrotransposition. *Proc. Natl Acad. Sci. USA*, **93**, 7767–7771.
42. Boutabout,M., Wilhelm,M. and Wilhelm,F.X. (2001) DNA synthesis fidelity by the reverse transcriptase of the yeast retrotransposon Ty1. *Nucleic Acids Res.*, **29**, 2217–2222.
43. Doulatov,S., Hodes,A., Dai,L., Mandhana,N., Liu,M., Deora,R., Simons,R.W., Zimmerly,S. and Miller,J.F. (2004) Tropism switching in Bordetella bacteriophage defines a family of diversity-generating retroelements. *Nature*, **431**, 476–481.
44. Harris,D., Kaushik,N., Pandey,P.K., Yadav,P.N. and Pandey,V.N. (1998) Functional analysis of amino acid residues constituting the dNTP binding pocket of HIV-1 reverse transcriptase. *J. Biol. Chem.*, **273**, 33624–33634.
45. Kaushik,N., Chowdhury,K., Pandey,V.N. and Modak,M.J. (2000) Valine of the YVDD motif of moloney murine leukemia virus reverse transcriptase: role in the fidelity of DNA synthesis. *Biochemistry*, **39**, 5155–5165.
46. Huang,H., Chopra,R., Verdine,G.L. and Harrison,S.C. (1998) Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. *Science*, **282**, 1669–1675.
47. Cases-Gonzalez,C.E. and Menendez-Arias,L. (2004) Increased G–>A transition frequencies displayed by primer grip mutants of human immunodeficiency virus type 1 reverse transcriptase. *J. Virol.*, **78**, 1012–1019.
48. Vartanian,J.P., Meyerhans,A., Asjo,B. and Wain-Hobson,S. (1991) Selection, recombination, and ----A hypermutation of human immunodeficiency virus type 1 genomes. *J. Virol.*, **65**, 1779–1788.
49. Blocker,F.J., Mohr,G., Conlan,L.H., Qi,L., Belfort,M. and Lambowitz,A.M. (2005) Domain structure and three-dimensional model of a group II intron-encoded reverse transcriptase. *RNA*, **11**, 14–28.
50. Cui,X., Matsuura,M., Wang,Q., Ma,H. and Lambowitz,A.M. (2004) A group II intron-encoded maturase functins preferentially in *cis* and requires both the reverse transcriptase and X domains to promote RNA splicing. *J. Mol. Biol.*, **340**, 211–231.