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The 3′ Ends of tRNA-Derived Short Interspersed Repetitive Elements Are Derived from the 3′ Ends of Long Interspersed Repetitive Elements

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Short interspersed repetitive elements (SINEs) are a type of retroposon, being members of a class of informational molecules that are amplified via cDNA intermediates and flow back into the host genome. In contrast to retroviruses and retrotransposons, SINEs do not encode the enzymes required for their amplification, such as reverse transcriptases, so they are presumed to borrow these enzymes from other sources. In the present study, we isolated a family of long interspersed repetitive elements (LINEs) from the turtle genome. The sequence of this family was found to be very similar to those of the avian CR1 family. To our surprise, the sequence at the 3′ end of the LINE in the turtle genome was nearly identical to that of a family of tortoise SINEs. Since CR1-like LINEs are widespread in birds and in many other reptiles, including the turtle, and since the tortoise SINEs are only found in vertical-necked turtles, it seems possible that the sequence at the 3′ end of the tortoise SINEs might have been generated by recombination with the CR1-like LINE in a common ancestor of vertical-necked turtles, after the divergence of side-necked turtles. We extended our observations to show that the 3′-end sequences of families of several tRNA-derived SINEs, such as the salmonid *Hpa*I family, the tobacco TS family, and the salmon *Sma*I family, might have originated from the respective LINEs. Since it appears reasonable that the recognition sites of LINEs for reverse transcriptase are located within their 3′-end sequences, these results provide the basis for a general scheme for the mechanism by which SINEs might acquire retropositional activity. We propose here that tRNA-derived SINEs might have been generated by a recombination event in which a strong-stop DNA with a primer tRNA, which is an intermediate in the replication of certain retroviruses and long terminal repeat retrotransposons, was directly integrated at the 3′ end of a LINE.

The reverse flow of genetic information from RNA back into DNA is known as retroposition, and each transposed informational element is known as a retroposon (23, 36). Highly repetitive elements in eukaryotic genomes (28, 29), such as short interspersed repetitive elements (SINEs), long interspersed repetitive elements (LINEs), and processed retropseudogenes, are all included in this category. Retroposition appears to be a major evolutionary force that has contributed to the maintenance of the remarkable fluidity of eukaryotic genomes (28, 36).

SINEs are short (approximately 80- to 400-bp) repetitive elements, which are often present at more 10^5 copies per genome. All SINEs reported to date, from sources as diverse as mammals and plants, are derived from tRNAs (20–22), with the exception of the primate *Alu* and rodent B1 families, which are derived from 7SL RNA (32, 35). The tRNA-derived SINEs are not simple pseudogenes for tRNAs but have a composite structure, with a region homologous to a tRNA, a tRNA-unrelated region, and an AT-rich region (20–22). Our group proposed recently that a possible origin of the region that is homologous to the tRNA of a SINE might be a primer tRNA, attached to a strong-stop DNA, which is an intermediate during the reverse transcription of certain retroviruses and long terminal repeat (LTR)-type retrotransposons (19). The source of enzymes for the reverse transcription of SINEs, however, remains to be determined.

Retroposons which encode a reverse transcriptase for replication of their genomes can be divided into three groups, namely, non-LTR-type retrotransposons (also known as LINEs, the designation used hereafter in this report), LTR-type retrotransposons, and retroviruses (5, 8). LTR-type retrotransposons are believed to have evolved from LINEs by gaining LTRs, and retroviruses are believed to have evolved from LTR-type retrotransposons by gaining *env* genes (5, 8).

Almost all LINEs, such as those of the chicken CR1 (for chicken repeat 1) family and the mammalian L1 family, are truncated at various positions in their 5′ regions, the lengths of which range from 100 to 1,000 bp. The presence of truncated forms suggests that a reverse transcriptase encoded by a LINE must recognize the 3′ end of the RNA template to initiate first-strand synthesis (4). This model was verified by Luan et al. in an elegant experiment with the R2Bm LINE of *Bombyx mori* (15).

The members of the CR1 family, which are present in avian genomes, have been characterized as LINEs. This family was first described as a SINE family in the chicken genome because most members of this family were extensively truncated at their 5′ ends and no obvious open reading frame was found (30). Subsequently, long members of the CR1 family were isolated, and it was shown that they were LINEs (2, 27). Vandergon and Reitman (33) detected sequences similar to the avian CR1 LINE in nonavian species, such as lizards and rays. In addition, they pointed out the similarity between the avian CR1 LINE and the tortoise SINE.

In the present report, we provide evidence for the sharing of

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the 3′-end sequence between the tortoise SINE and a CR1-like LINE from turtles. Furthermore, we demonstrate that tRNA-derived SINEs may generally have 3′ ends in common with particular LINEs. From our results, we propose a model for the molecular mechanism by which tRNA-derived SINEs acquired retropositional activity during evolution.

MATERIALS AND METHODS

DNA samples. Genomic DNAs from *Platemys spixii* (side-necked turtle), *Eretmochelys imbricata* (sea turtle), *Apalone ferox* (soft-shelled turtle), *Chinemys reevesi* (tortoise), *Caiman crocodilus* (caiman), *Gallus gallus* (chicken), *Homo sapiens* (human), and *Anguilla japonica* (eel) were extracted as described elsewhere (1).

Dot blot analysis. Progressively decreasing amounts of total genomic DNA (1 μg, 200 ng, and 40 ng) from individuals of several species were dotted on a membrane. In one case, progressively decreasing amounts of cloned DNA (10 ng, 2 ng, and 400 pg) containing the tortoise Pol III/SINE (TE6) (6) were dotted on the membrane as a control. Probe 1 was prepared as follows. DNA fragments of approximately 150 bp were amplified by PCR with genomic DNA from the tortoise as a template and with TEF1 and TER1 as primers (positions 1 to 154 in Fig. 4). The fragments were isolated from an agarose gel and then labeled internally, using the same primers (TEF1 and TER1), with [α-^{32}P]dCTP. Hybridization was carried out at 42°C in 50% formamide. Washing was performed at 55°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate (SDS). In the case of probe 2, the 5′ end of the oligonucleotide (PROTO-R1) was labeled with [γ-^{32}P]ATP. Hybridization was carried out at 48°C without formamide, and washing was performed at 55°C in 0.3× SSC–0.1% SDS. The nucleotide sequence of PROTO-R1 was 5′-CCCCAG(A/T)T(G/C)C(T/C)TA(A/C)(G/A)TGGCCCCCTCAAGGA-3′ (positions 89 to 60 in Fig. 4).

DNA cloning and sequencing. Genomic libraries were constructed by ligation of λgt10 arms with genomic DNA of *P. spixii* or *Apalone ferox* that had been completely digested with *Eco*RI. Probe 1, which had been labeled internally with [α-^{32}P]dCTP, was used as the probe for isolation of phage clones from the libraries. Respective inserts of positive clones were subcloned in the pUC vector and then sequenced by the dideoxy chain termination method (26).

Analysis by PCR. PCR (25) was performed with two sets of primers specific to the CR1 element, CRF1 plus CRR0 and CRF2 plus CRR1, and two sets of primers specific to the SINE, TEF1 plus TER0 and TEF1 plus PROTO-R1. Ten nanograms of total genomic DNA from each species and 100 pg or 1 ng of cloned DNA were used as templates. Each reaction was performed under the following conditions: 1 cycle of 93°C for 2 min, 55°C for 1 min, and 72°C for 10 s and 29 cycles of 93°C for 1 min, 55°C for 1 min and 72°C for 10 s. The nucleotide sequences of primers used in the experiments were as follows: CRF1, 5′-GAG(G/A)T(T/C)TAGGTTGGA(T/C)ATTAGG-3′ (positions −229 to −208 in Fig. 3); CRR0, 5′-ATTAGGGTTGGAAGGGACCT-3′ (positions −16 to −35 in Fig. 3); CRF2, 5′-GAGGGTGGTGA(G/A)GCACTGGAA-3′ (positions −190 to −170 in Fig. 3); CRR1, 5′-TCATCTAGTCCAACCCCCTGCT-3′ (positions −43 to −64 in Fig. 3); TEF1, 5′-GGGAGGGATAGCTCAGTGGT-3′ (positions 1 to 20 in Fig. 4); TER0, 5′-ATAT(G/A)CC(A/T)ATCTCCTAG AAC-3′ (positions 174 to 155 in Fig. 4); and TER1, 5′-TGGAA(G/A)GGACC T(T/C)(G/A)(G/A)(A/C)AGG-3′ (positions 154 to 136 in Fig. 4).

Genomic DNA walking. Five hundred nanograms of eel genomic DNA, which had been completely digested with *Eco*RI, was ligated with 50 ng (3.3 pmol) of an *Eco*RI cassette (Takara, Shiga, Japan). With one-fifth of the sample as a template and oligonucleotides C1 and Fok4 as primers, the first PCR was performed at 55°C (annealing) for 30 cycles. With 1/100 of the product of the first PCR as a template and oligonucleotides C2 and Sma5E as primers, the second PCR was performed at 55°C (annealing) for 30 cycles. The product of the second PCR was digested with *Eco*RI and then ligated into the pUC18 vector, and then the nucleotide sequences of the cloned DNAs were determined. The nucleotide sequences of the primers used in the experiments were as follows: C1, 5′-GTACATATTGTCGTTAGAACGCG-3′; C2, 5′-TAATACGACTCACTATA GGGAGA-3′; Fok4, 5′-TTACATTTAAGTCATTTAGCAG-3′; and Sma5E, 5′-TCGGAATTCTGCCATTTAGCAGACGCTTT-3′.

Nucleotide sequence accession numbers. The nucleotide sequences of the turtle LINEs reported in this article have been deposited in the DDBJ, EMBL, and GenBank nucleotide databases with accession numbers D82938, D82939, and D82940.

RESULTS

A CR1-like LINE family is present in the genome of a primitive turtle. Living turtles can be divided into two major suborders, namely, Cryptodira, which includes vertical-necked turtles, and Pleurodira, which includes side-necked turtles (Fig. 1). The suborder Cryptodira consists of several superfamilies, such as Testudinoidea (tortoises), Trionychoidea (soft-shelled turtles), and Chelonioidea (sea turtles) (9). The fossil record suggests that the suborder Pleurodira diverged from the suborder Cryptodira about 200 million years ago (24). In previous studies, a family of SINEs, designated the tortoise Pol III/SINE, was isolated from a tortoise in the superfamily Testudinoidea, and they were characterized as members of a typical tRNA-derived SINE family, each member of which consists of three regions, namely, a tRNA^{Lys}-related region, a tRNA-unrelated region, and an AT-rich region (Fig. 2A) (6). To examine the distribution of the tortoise Pol III/SINE in living turtles and other species, we performed a dot hybridization experiment. In the first experiment, almost the entire sequence of this SINE was used as a probe (probe 1 in Fig. 2A). Signals were detected in all turtles, including a side-necked turtle, and

Order	Suborder	Superfamily
Testudines (turtles)	Cryptodira (vertical-necked turtles)	Testudinoidea (tortoises) Trionychoidea (soft-shelled turtles) Chelonioidea (sea turtles)
	Pleurodira (side-necked turtles)	

FIG. 1. Classification of living turtles.

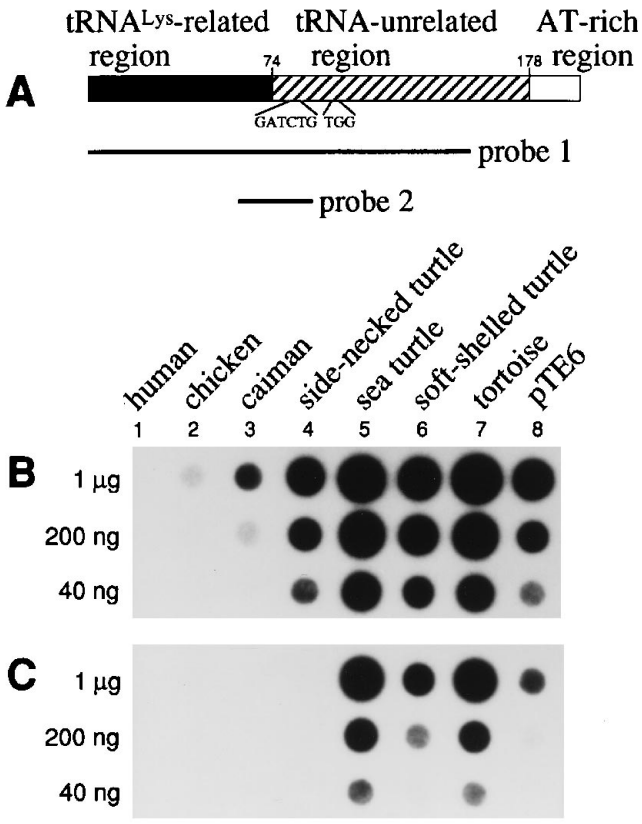

FIG. 2. Distribution of the tortoise Pol III/SINE-related sequence in turtles and their living relatives. (A) Schematic representation of the composite structure of the tortoise Pol III/SINE and indication of the regions used as probes. (B and C) Dot hybridization experiments were performed with probe 1 (B) and probe 2 (C). Progressively decreasing amounts of total DNA (1 μg, 200 ng, and 40 ng) from individuals of the indicated species were dotted on a membrane (columns 1 to 7). In column 8, progressively decreasing amounts of cloned DNA (10 ng, 2 ng, and 400 pg) of the tortoise Pol III/SINE (TE6) (6) were dotted on the membrane as a control.

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                      -360                -340                -320                -300                -280
CR1 (chicken) CCTGGAGAAGAGAAGGCTGCGCGGTGACCTCATTGCAGCCTTTCAGTACCTGAAGGGAGCCTATAAACAGGAAGGGAGTAAACTCTTTGAAAGGGTAGAT
               *** **********  ** * ** **  * ** ********* * ******** ** *  *  *   **** **   *** ** ***  *  *** *
CONSE(Ps)      CTGCAGAAGAGARRAATGAGGGGGGATTTGATAGCAGCCTTTAACTAYCYRAAAGGGGGTTCCAGGGAGGATGGAGCTARGCTGYTTTCAGWGGTGRCA
4-2(Ps)       T••••••••••••GG•••••••••••••••••••••••••••••••••T•TG••••••••••••••••••••••••••••G••••C••••••T••••A••
4-1(Ps)        ••••••••••••AA•••••••••••••••••••••••••••••••••C•CA••••••••••••••••••••••••••••A••••T••••••A••••G••

                      -260                -240                -220                -200                -180
CR1 (chicken) AACAGCAGGACAAGGGGGAACGGTTTTAAGTTGAAAGAGGGAAGATTTAGGTTGGATGTTAGGGGGAAGTTCTTTACCAGGAGAGTGGTGAGGTGCTGGA
               *   *** ****** * ** *** * ****** *   *** ** * *********  *****   ***** ** ** ***** ******* *  *****
CONSE(Ps)     GATGACAGAACAAGGARCAATGGTCTCAAGTTGCAGTGGGGGAGGTCTAGGTTGGACATTAGGAAAAAGTTTTTCACTAGGAGGGTGGTGAAGCACTGGA
4-2(Ps)       ••••••••••••••••G•••••••••••••••••••••••••••••••••••••••••••••••••c••••••••••••••••a••••••••••••••••
4-1(Ps)       ••••••••••••••••A•••••••••••••••••••••••c••••••••••••••••••••••••••••••••••••••••••••••••••••••t••••
2-3(Ps)       tctgacagcggtctataaaatcatttttgaatgaattg•t••••••••••••••••t••••••g•••ca•••••••••••••••••••••••••••••••

                      -160                -140                -120                   -100                -80
CR1 (chicken) ACAGGCTGCCCAGAGAGGTTGTGGATGCTCTGTCCCTGGAGGTGTTCAAGGCCAGGTTGGATGGGGCCCTGGGCAACCTGGTCTAGTAAATGGGGATGTT
              *  ** * ** ** ***** *****  ***  **  * ***** ** ***    ** * **     ****       ****  *  *  * *  ** ***
CONSE(Ps)     ATGGGTTACCTAGGGAGGTGGTGGAATCTCCATCTTTAGAGGTTTTTAAGCTGCGGCTTGACAAAACCCT-----CGCTGGGATGATTTA-GCTGAGGTT
4-2(Ps)       ••••••••••c•••••t••••••••••••••••••••••••••••••••••••••••••••••••••ta•-----••c•••••••••g••-a••••-ta•
4-1(Ps)       ••a•••••••••••••••••••••c•••••t•-•c•••••••••a•••••••••••••••••••••••••-----•••••••••••••••-•••ag••••
2-3(Ps)       •••t••••••••••••••••••••••••••••••••••a••••••••••••••••••••••••••g••••-----g••••••••••••••-•••••••••

                     -60                  -40                  -20
CR1 (chicken) GGTGGCCCTGCCCAGCAGGGGGGTTGGA-GATTCGTGATCCTCGAGGTCCCTTCCAACCCAGGCCATTCTGTGATTCTGT
               **    **    ********* *****  *   *   **** *************** *     ***** ******* *
CONSE(Ps)     AGTCCTGCT-TTGAGCAGGGGG-TTGGACTAGATGACCTCCT-GAGGTCCCTTCCAACTC-TAATATTCTATGATTCTAT
4-2(Ps)       •••••••••-••••••••••••-•••••••••••••••••••-•••••••••••••••••-•••••••••••••••••••aaagaattc
4-1(Ps)       g••••••••-••••••••••••-••••••••••••••••t••-••••••••••••••t••-•••••••••••••••••c•gacatggcatgccacatccg
2-3(Ps)       •••••••••-••••••••••••-•••••••••••••••••••-•••••••••••••••••-•••••••••••t••••••aaagggaatagggaattgtta
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FIG. 3. A CR1-like LINE family was found in the side-necked turtle. Several clones [4-2(Ps), 4-1(Ps), and 2-3(Ps)] were isolated from the turtle with probe 1 (see text), and their sequences were determined. A consensus sequence of these clones [CONSE(Ps)] was aligned with the sequence of chicken CR1 (2). The nucleotides are numbered from the 3′ terminus, as is generally the custom for LINEs. Asterisks indicate the nucleotides common to the consensus sequences of the chicken CR1 LINE and the turtle CR1-like LINE. Dots indicate nucleotides identical to those of the consensus sequence of the turtle CR1-like LINE. Gaps (–) have been introduced to maximize homology.

in a caiman. In addition, a faint signal was observed in a chicken (Fig. 2B). When we used just part of the SINE as the probe, signals were detected only in turtles other than the side-necked turtle (Fig. 2C).

Our observations prompted us to try to explain the difference in hybridization signals. We constructed a genomic library for a side-necked turtle (*P. spixii*). Using probe 1, we isolated several clones from this library and determined their sequences (Fig. 3). To our surprise, these clones showed remarkable similarity to the chicken CR1 element. One clone, designated 4-2(Ps), had extensive similarity to chicken CR1 in the region of the 2.1-kbp *Eco*RI fragment (64% similarity over the entire 2.1 kbp). Two other clones, designated 2-3(Ps) and 4-1(Ps), had a common sequence at their 3′ ends, and their 5′ regions were truncated, as is the case for several LINEs, including the chicken CR1 element (2, 8) (see the introduction).

The 3′ ends of the CR1-like LINE and the tortoise SINE are similar. The experiment described above also revealed that a region of 80 bp at the 3′ end of the CR1-like LINE of the side-necked turtle was almost the same as the 3′ end of the tortoise Pol III/SINE isolated from *Chinemys reevesi* (previously known as *Geoclemys reevesi*) (6). This similarity explains why CR1-like LINEs could be isolated from a genomic library of the side-necked turtle with the tortoise SINE as the probe (probe 1). To confirm the presence of the tortoise SINE in the superfamily Trionychoidea, as suggested by the dot hybridization experiment with probe 2 (Fig. 2C), we made a genomic library of a soft-shelled turtle (*Apalone ferox*) and isolated several clones. Each clone contained the repetitive unit of the tortoise Pol III/SINE, and the flanking sequences of the respective units were completely different from one another, confirming the presence of the tortoise Pol III/SINE in turtles other than the tortoise, as well as the sharing of the 3′-end sequence between members of the SINE family and the CR1-like LINE. Figure 4 shows an alignment of a consensus sequence of the CR1-like LINE, designated CR1(Ps), and consensus sequences of the SINE(Cr) of the tortoise and the SINE(Af) of the soft-shelled turtle. The sequence of the SINE(Af) family was the same as that of the tortoise SINE(Cr) except for the insertion of 16 bp at position 97. The tRNA-unrelated region of the SINE from position 97 to 176 was very similar to a region of about 80 bp at the 3′ end of the CR1-like element (positions −78 to −1), whereas the $tRNA^{Lys}$-related region (underlined in Fig. 4) and the adjacent 20 bp of the tRNA-unrelated region in the SINE exhibited no obvious similarities. In particular, the 3′-end sequence of one member of the SINE(Cr) family, designated TE3 (6), exhibited considerable similarity (90%) to that of the CR1-like LINE. TE3 has been proposed to belong to an ancient group on the basis of the sequence divergence from other members of the SINE(Cr) family (6).

CR1-like LINEs are present in all living reptiles and birds, whereas the tortoise Pol III/SINE is confined to cryptodires, a dominant group of living turtles. The results of dot hybridization experiments, as shown in Fig. 2, suggested that CR1-like LINEs might be distributed in a wide range of species. However, several questions remained unanswered, as follows. (i) Are CR1-like LINEs present in all cryptodires? (ii) Do crocodilians have CR1-like LINEs? (iii) Is the tortoise Pol III/SINE completely missing from side-necked turtles? To answer these questions, we performed analyses by PCR with several sets of primers specific to the SINE and to CR1 (Fig. 5). As shown in Fig. 5A, DNA products of both 213 and 147 bp, specific to the CR1-like LINE, were detected in the case of chickens, caimans (which are crocodilians), and side-necked turtles, as well as in all cryptodires tested. This result suggested to us that CR1-like LINEs might be ubiquitous in all living reptiles and birds. We failed to detect any signals with human

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                -180                -160                -140                -120                -100
CR1(Ps)  GAGGGTGGTGAAGCACTGGAATGGGTTACCTAGGGAGGTGGTGGAATCTCCATCTTTAGAGGTTTTTAAGCTGCGGCTTGACAAAACCCTCGCTGG
         * * * * *    ** ***  *  *          *           *     ** **  **      *    *  *  *      *
TE3      GGGAGGGATAGCTCAGTGGTTTAAGCATTGGCCTGCTAAACCCAGGGTTGTGAGTTCAGTCCTTGAGGGGGCCATGTAAGCAACTGGGGTAAAAAA
         ********************** ********************************** ***************  ** * * ****** ******
SINE(Cr) GGGAGGGATAGCTCAGTGGTTTGAGCATTGGCCTGCTAAACCCAGGGTTGTGAGTTCAATCCTTGAGGGGGCCACTTAGGGATCTGGGGCAAAAAA
         ************************************************************  * ***** ******** ***********
SINE(Af) GGGAGGGATAGCTCAGTGGTTTGAGCATTGGCCTGCTAAACCCAGGGTTGTGAGTTCAATCCTTGCAGAGGCCATTTAGGGATTTGGGGCAAAAAT
                    20                  40                  60                  80

                -80                 -60                 -40                 -20
CR1(Ps)  GATGATTTAGCTGAGGTTAGTCCTGCTT-TGA--GCAGGGGGTTGGACTAGATGACCTCCTGAGGTCCCTTCCAACTCTAATATTCTATGAT-TCTAT
           * *  *** **** ********* ***  *** ********************* ********* ******* ** *** * ****** ****
TE3      -----TCTGTCTGGGGTTGGTCCTGCTT-TGA--GCA-GGGGTTGGACTAGATGACCTGCTGAGGTCCTTTCCAACCCTGATAGTTTATGAT-TCTAAGACTGGA
                          ***********  ***  *** **** ****** ********    ************    **   ** **   ** ***   * *
SINE(Cr) ----------------TTGGTCCTGCTAGTGAAGGCAGGGGGCTGGACTCGATGACCTTTCAAGGTCCYTTCCAGTTCTAGGAGATTGGTATATCTCCAATTATT
                          *********** ************* ******** ********************************** ** * ******* *****
SINE(Af) TTGTCAGGGATGGTACTTGGTCCTGCT-GTGAAGGCAGGGGACTGGACTCAATGACCTTTCAAGGTCCCTTCCAGTTCTAGGAGATAGGCA-ATCTCCA-TTATT
                          100                 120                 140                 160                 180
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FIG. 4. The 3′-end structure is shared by the turtle CR1-like LINE and the tortoise Pol III/SINE. The consensus sequence of the CR1-like LINE [CR1(Ps)] is compared with those of the Pol III/SINE from a tortoise [SINE(Cr)] and from a soft-shelled turtle [SINE(Af)]. One individual member of the SINE(Cr) family (TE3) is included. Identical nucleotides are indicated by asterisks. The $tRNA^{Lys}$-related regions are underlined. The nucleotides in SINEs are numbered from the 5′ terminus, whereas the nucleotides in CR1(Ps) are numbered from the 3′ terminus.

DNA, although mammals are believed to be the closest living relatives of reptiles and birds (10). This result indicates that the CR1-like LINEs might have arisen in a common ancestor of reptiles and birds after divergence of the mammalian lineage (Fig. 6).

PCR products of 174 or 89 bp, specific to the tortoise Pol III/SINE, were detected only with DNA templates from cryptodires, namely, a sea turtle, a soft-shelled turtle, and a tortoise, and they were not detected in the case of the side-necked turtle (Fig. 5B). This result suggests that the tortoise Pol III/SINE emerged in a common ancestor of cryptodires after the divergence of pleurodires (Fig. 6). It also suggests that the composite structure of the tortoise SINE, which contains part of a LINE sequence, appeared at the time of the first generation of the tortoise Pol III/SINE (TER0 and TER1, primers that were used to detect the SINE in Fig. 5B, are complementary to the 3′-terminal region of the SINE that is homologous to the CR1 LINE). This hypothesis raises the additional question of the origin of the 5′ half of the tortoise Pol III/SINE and the way it was combined with the 3′ end of the CR1-like LINE (see Discussion).

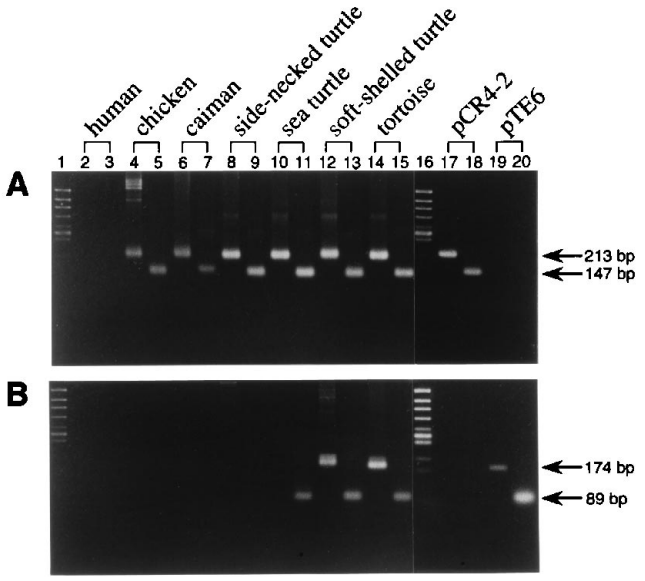

FIG. 5. The CR1-like LINE is present in all of the reptiles examined and in a bird, whereas the tortoise Pol III/SINE is confined to a dominant group of living turtles. PCR analysis was performed with two sets of primers specific to the CR1 LINE, namely, CRF1 plus CRR0 and CRF2 plus CRR1 (A), and with two sets of primers specific to the SINE, namely, TEF1 plus TER0 and TEF1 plus PROTO-R1 (B). Ten nanograms of total DNA from each species (lanes 2 to 15) and 100 pg (A) or 1 ng (B) of cloned DNA (lanes 17 to 20) were used as templates. Products of PCR that were amplified with each set of primers are indicated by arrows. Although in lane 10 of panel B, no product of 174 bp is visible, a product of the expected size (154 bp) was detected when another primer, designated TER1, which started 20 bp upstream of TER0, was used (data not shown). Lanes 1 and 16 show size markers (*Hin*cII digest of ϕX174 DNA).

Other SINEs, such as the salmonid *Hpa*I, the tobacco TS, and the salmon *Sma*I SINEs, also have a sequence at the 3′ end that is common to the respective LINE or LINE-like sequence. To examine whether members of a SINE family generally have a fused structure, with a certain LINE at the 3′ end, we searched in the EMBL database (release 40.0) for examples other than the pair composed of the tortoise Pol III/SINE and the CR1-like LINE. Figure 7A shows an alignment of consensus sequences of a SINE family, designated the salmonid *Hpa*I family, and a LINE family from salmonid fishes, known as the RSg-1 family. The *Hpa*I family is a member of the superfamily of tRNA-derived SINEs (11) and is found in many salmonid species (12, 13). The 3′-end sequence of the salmonid *Hpa*I

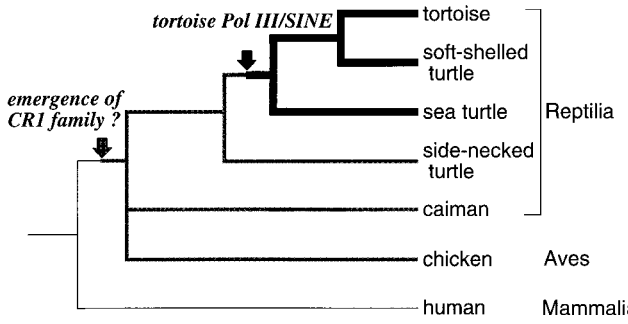

FIG. 6. Timing of the appearance during evolution of the CR1-like LINE and of the tortoise Pol III/SINE. Phylogenetic relationships among amniota are taken from the work of Hedges (10).

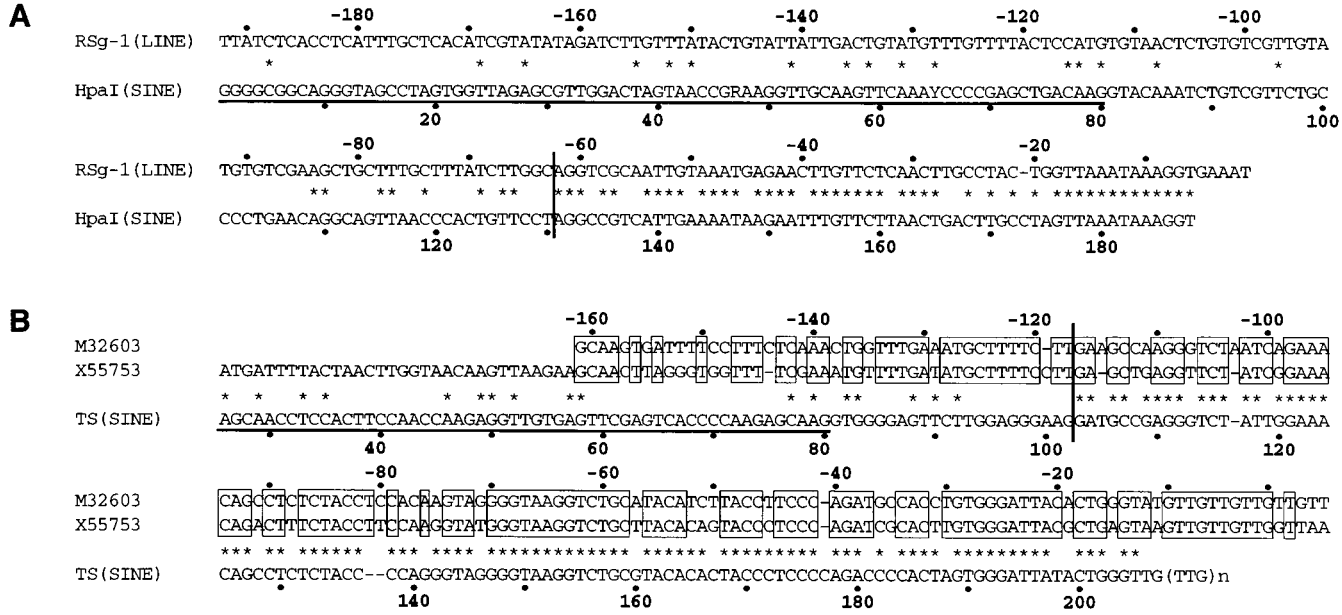

FIG. 7. tRNA-derived SINEs other than the tortoise SINE also have a composite structure, being fused with a LINE or a LINE-like sequence at their 3′ ends. (A) Alignment of consensus sequences of the salmonid *Hpa*I SINE (12) and the RSg-1 LINE from rainbow trout (37). Identical nucleotides are indicated by asterisks. The tRNA-related region of the *Hpa*I SINE is underlined. Numbering systems are as in Fig. 4. The junction (see text) is shown by a vertical line. (B) Comparison of the sequences of LINE-like elements from solanaceous plants (accession numbers M32603 and X55753) with the consensus sequence of the tobacco TS SINEs (39). Identical nucleotides in these two LINE-like sequences are boxed. Identical nucleotides in the sequence with accession number X55753 and TS SINE are indicated by asterisks. The junction (see text) is shown by a vertical line.

family (positions 131 to 188 in Fig. 7A) was found to have significant homology to approximately the last 60 bp of members of the RSg-1 LINE family (positions −62 to −6). The RSg-1 family, whose members have well-defined 3′ ends that contain poly(A) segments and heterogeneous 5′ ends, was characterized as a LINE family in the rainbow trout (37).

The TS family is a SINE family that has been characterized in the tobacco genome (39). Various plants belonging to the families Solanaceae and Convolvulaceae contain sequences homologous to the TS family (39). Using the 3′-end sequence of the TS family as a probe, we made another search of the EMBL database. We found two sequences that showed significant similarity to each other over a region of 160 nucleotides (77% [Fig. 7B]). One sequence was found in the second intron of a tomato protease gene (accession number M32603), and the other was found in the second intron of a potato actin gene (accession number X55753). The nucleotides common to these two sequences are boxed in Fig. 7B. We found that the two sequences have a sequence common to the 3′-end sequence of the TS family (positions −117 to −13), whereas their upstream sequences (positions −161 to −118) exhibit no obvious similarity to the TS family. Although these sequences have not been reported to be LINEs, they suggest the presence of a family of LINEs that have 3′ ends in common with the TS SINE in the genomes of plants in the family Solanaceae.

The salmon *Sma*I family is a family of SINEs, the members of which are present in the genomes of only two species, namely, the chum and the pink salmon, in the genus *Oncorhynchus* (11). When we used the 3′-end sequence of the *Sma*I family as a probe for a homology search, we found a highly homologous sequence within a pseudogene for an immunoglobulin heavy chain from the tenpounder, a primitive teleost fish (accession number M29586). The sequence was found to be homologous to approximately the last 40 nucleotides of the salmon *Sma*I family (94% identity), whereas the sequence upstream of the homologous sequence was found to show no obvious similarity to the *Sma*I family. We postulated that a LINE family with a 3′ end common to the salmon *Sma*I family must exist in the genomes of teleost fishes, and we tried to isolate such elements (Fig. 8). We employed the method of genomic DNA walking that is known as cassette PCR. First, the genomic DNA of an eel was digested with *Eco*RI, and *Eco*RI cassettes were ligated to the fragments. Using oligonucleotides complementary to the cassette and those complementary to the 3′-end sequence of the *Sma*I family as primers, we performed PCR. Products of PCR of various lengths were cloned, and their sequences were determined (Fig. 8A). All of the clones were found to have a common sequence at their 3′ ends, whereas their 5′-upstream sequences were found to be truncated at various positions. Southern blot analysis with genomic DNA from the eel revealed that these elements were highly repetitive and were dispersed within the genome (data not shown). The elements appeared not to belong to the *Sma*I SINE family, because we failed to detect any products of PCR with eel DNA and several sets of primers specific for the *Sma*I family (data not shown). Therefore, it seems likely that these elements are members of a LINE family in eels. However, long forms of such elements must be isolated from the eel genome to validate this hypothesis. The consensus sequence of the eel LINE-like elements (positions −38 to −21) was found to show significant similarity (94% identity) to the 3′ region of the salmon *Sma*I family (positions 103 to 120), whereas the upstream sequences were found to show no obvious similarity to one another (Fig. 8B), as was the case for other pairs of SINEs and LINEs. The char *Fok*I family is a SINE family whose members are present in all species of the genus *Salvelinus* (11). The 3′-end sequences of the *Fok*I and *Sma*I families are almost the same (11), a correspondence that suggests that they might have been generated in a similar manner during evolution (see Discussion).

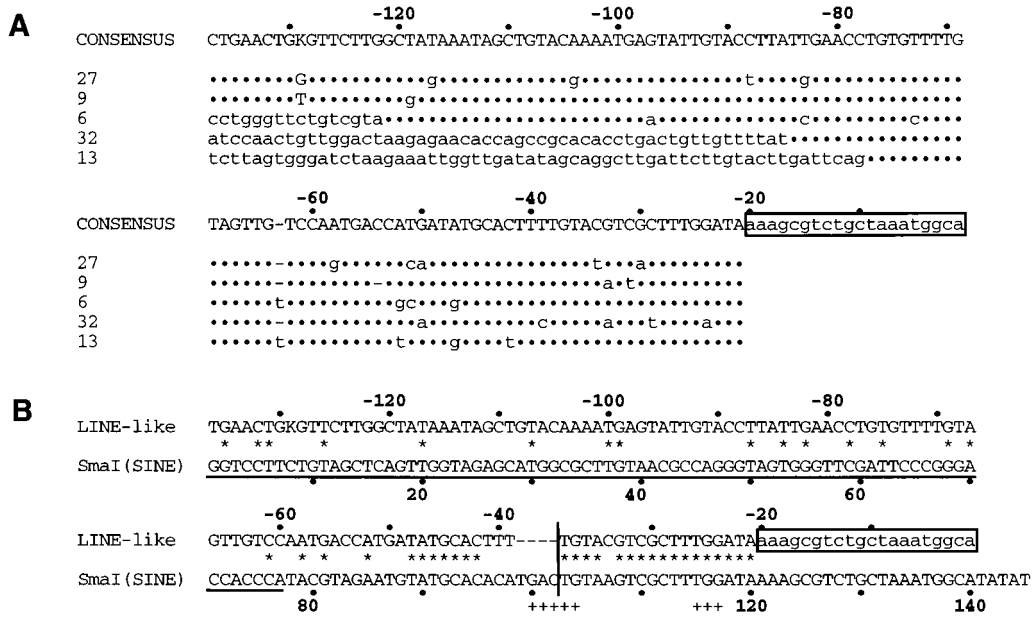

FIG. 8. LINE-like elements in eels show significant similarity to the salmon *Sma*I SINE at their 3′ ends. (A) Sequences of members of the LINE-like elements isolated from the eel genome are aligned. The oligonucleotide sequence complementary to the *Sma*I SINE was used as a primer for cassette PCR (see text). The box indicates the sequence complementary to the primer (Sma5E). Dots indicate nucleotides identical to those in the consensus sequence. (B) Alignment of the consensus sequences of the LINE-like elements of the eel and salmon *Sma*I SINEs (11). Identical nucleotides are indicated by asterisks. The $tRNA^{Lys}$-related region of the *Sma*I SINE is underlined. Numbering systems are as in Fig. 4. The junction is shown by a vertical line (see text). The junction is located within two conserved motifs, namely, GA(T)CTG and TGG (plus signs), which were identified in the $tRNA^{Lys}$-related SINE superfamily (19).

DISCUSSION

tRNA-derived SINEs might have been generated by fusion with the 3′ ends of respective LINEs. In the present study, we demonstrated that the sequences at the 3′ end of the tortoise Pol III/SINE and the CR1-like LINE are shared (Fig. 4). We showed previously that transcription in vitro of total genomic DNA of the tortoise resulted in one major Pol III-transcribed RNA with a discrete size of about 180 nucleotides (6, 7). Fingerprinting analysis indicated that this RNA represented transcripts of members of the tortoise Pol III/SINE in the tortoise genome (6, 7). Therefore, the composite structure of the tortoise SINE fused with part of a LINE is representative of a majority of members of this SINE, and it is not a rare example of a member of this SINE family.

Similar considerations can be applied to the case of the salmonid *Hpa*I SINE (Fig. 7A). We recently compiled 99 sequences of members of the *Hpa*I SINE family and proposed an evolutionary history for the active sequences in this family by dividing the members into 12 subfamilies on the basis of changes in diagnostic nucleotides (13). Therefore, the fused structure of the *Hpa*I SINE can be considered to represent a SINE family that was subjected to retroposition during evolution.

In the cases of the tobacco TS SINE and the salmon *Sma*I SINE, the generality of the composite structure of a SINE fused to part of a LINE was suggested by the presence of LINE-like sequences in the genomes of solanaceous plants and eels (Fig. 7B and 8). Although this result must be confirmed by future studies, we propose that the fused structure of tRNA-derived SINEs might be a general phenomenon, for the reasons given in the next section.

Enzymes required for the retroposition of tRNA-derived SINEs might be provided by the respective LINEs. Luan et al. have provided a biochemical demonstration of a mechanism for the retroposition of R2, a LINE family in *B. mori* (15). This mechanism is referred to as target DNA-primed reverse transcription. The R2 protein, which has both sequence-specific endonucleolytic and reverse transcriptase activities, makes a specific nick in one of the DNA strands at the insertion site and uses the 3′-hydroxyl group that is exposed by this nick to prime the reverse transcription of its RNA transcript. Thus, the R2 protein can recognize specifically the sequence near the 3′ end of the RNA transcript for initiation of first-strand synthesis. Furthermore, Luan and Eickbush found that the last 250 nucleotides that correspond to the 3′ untranslated region of the R2 transcript are critical for the reaction (14). These authors (15) also proposed that a target DNA-primed mechanism of reverse transcription, similar to that involving R2, might be generally applicable to other LINEs. Moreover, they suggested that a similar mechanism might also be operative in the case of SINEs, since SINEs, as well as LINEs, do not have LTRs, which are required for efficient reverse transcription, in the 3′-terminal regions of SINEs. Recently, Zimmerly et al. demonstrated that the reverse transcription of a yeast mitochondrial DNA group II intron was accomplished by an analogous target DNA-primed reverse transcription (40), suggesting that the mechanism might be of more general relevance than had been proposed previously.

In the present study, we demonstrated that the 3′ end of the tRNA-unrelated region of the tortoise Pol III/SINE is actually identical to the 3′-end region of the CR1-like LINE in the turtle genome. This identity suggests that the enzymatic machinery responsible for the retroposition of the tortoise Pol III/SINE might also be the same as that responsible for the retrotransposition that involves the CR1-like LINE. During the course of our study, Vandergon and Reiman (33) reported the similarity between the chicken CR1 and the tortoise Pol III/SINE (see the introduction). We have extended their observations to demonstrate that tRNA-derived SINEs might be generally composed of a chimeric structure, with a tRNA-

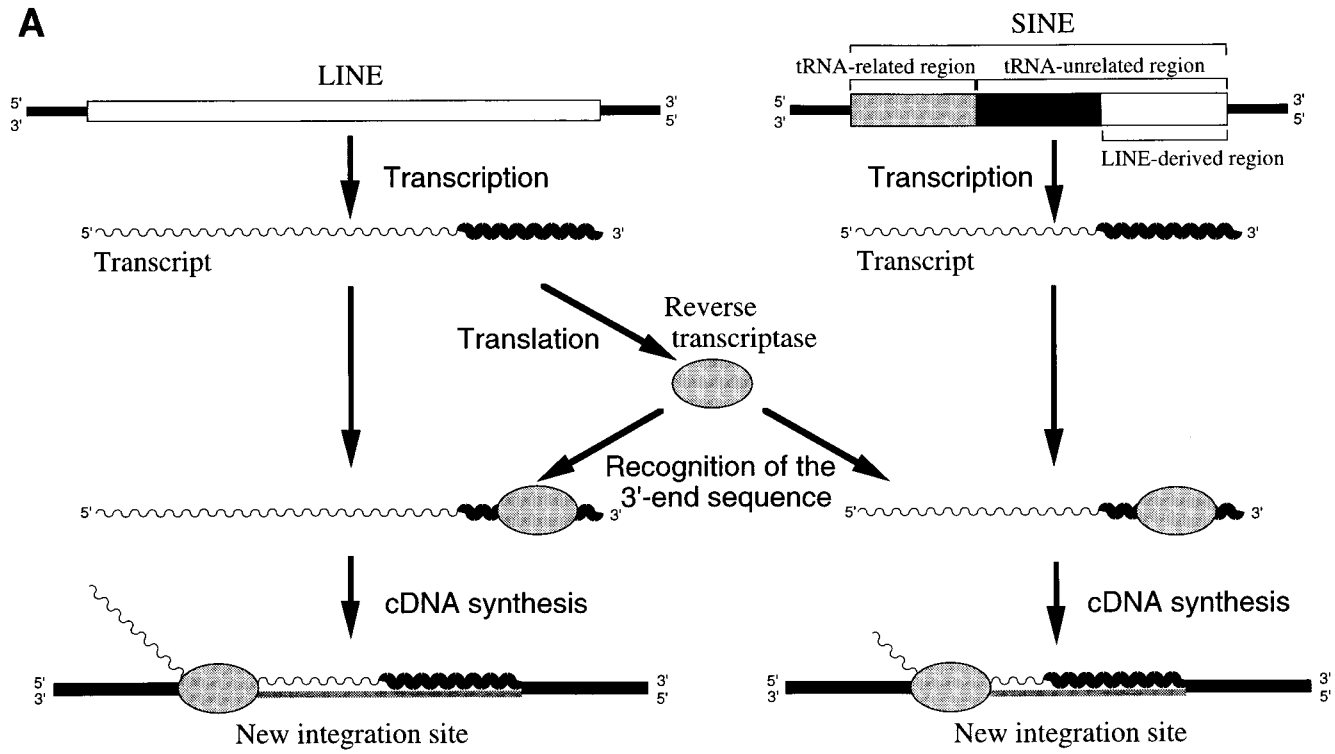

FIG. 9. (A) Enzymes required for the retroposition of a tRNA-derived SINE might be provided by a corresponding LINE. Transcripts of the SINE are recognized, through the common tail sequence, by a reverse transcriptase generated from the corresponding LINE, and they are reverse transcribed into cDNA by the target DNA-primed mechanism (15) adopted by LINEs. (B) A possible model for the initial generation of tRNA-derived SINEs. In this model, a strong-stop DNA with a primer tRNA (19) was integrated into the 3′ portion of a LINE, which gave rise to a primordial SINE.

related region plus the left half of the tRNA-unrelated region and the right half of the tRNA-unrelated region which is homologous to the 3′ end of a LINE. Two complete examples of this organization are provided by the tortoise Pol III/SINE and the salmonid *Hpa*I family, and two probable examples are provided by the tobacco TS family and the salmon *Sma*I family. The results strongly suggest that each SINE family recruited the enzymatic machinery for retroposition from the corresponding LINE through a common "tail" sequence. This scenario is shown schematically in Figure 9A. The model predicts that when there is a simple tRNA-derived SINE family, a LINE family, which has the same 3′ end as the tRNA-derived SINE family, must always be present in the genome of the same organism.

The mechanisms of retroposition of SINEs and LINEs might not necessarily be the same. We found that SINEs and LINEs had similar 3′ tails in common. It should be noted, however, that the 3′-terminal repeats, which follow these tails of SINEs and LINEs, often differ between the SINEs and LINEs. In general, while LINEs tend to have simple repeats of A residues at the 3′ end (4), SINEs appear to have more-complex repeats, such as $(AAATGT)_n$ in the char *Fok*I family (11) and $(TTG)_n$ in the tobacco TS family (39).

The molecular mechanisms responsible for the different 3′-terminal repeats are unknown. LINEs are known to be transcribed by RNA polymerase II via the internal promoters that are characteristic of this group (18, 31), whereas SINEs are transcribed by RNA polymerase III via the internal promoters of tRNA-related regions (16). Therefore, it is possible that distinct mechanisms for termination of the two transcriptional systems might be responsible for the differences in the 3′-terminal repeats. LINEs might have pure poly(A) repeats because these were generated and regenerated by the polyadenylation apparatus (34). By contrast, in the case of many tRNA-derived SINEs, RNAs of discrete sizes can be transcribed in vitro by RNA polymerase III, and therefore, these SINEs are considered to include novel termination signals (17). The way in which the 3′-terminal repeats of these SINEs were generated remains to be determined.

A possible mechanism for the initial generation of tRNA-derived SINEs. tRNA-derived SINEs can be classified into a few superfamilies by reference to the tRNA species from which they originated, and $tRNA^{Lys}$ is the most common source of SINEs (20–22). Thus, for example, the $tRNA^{Lys}$-related SINE superfamily includes the tortoise Pol III/SINE, the rodent type 2 family, the squid SK family, the salmon *Sma*I family, and the char *Fok*I family (20–22). We recently proposed a model to explain the way in which the tRNA-related regions of these SINEs might have been generated during evolution (19). Our model is based on the following observations. First, the tRNA-related regions of these five families of SINEs end with the sequence CCA. The CCA sequence is added posttranscriptionally to tRNAs and is not encoded by their genes. Accordingly, it seems plausible that the $tRNA^{Lys}$-related regions of these SINEs might have been derived from mature $tRNA^{Lys}$ itself. Therefore, we have to postulate a mechanism for the extension of DNA beyond the end of the CCA sequence of $tRNA^{Lys}$ in order to explain the origin of the composite structure of SINEs. This requirement brings to mind the LTR-based mechanism of reverse transcription of retroviruses. The second observation is that conserved DNA segments, namely, GATCTG and TGG, at a distance of 10 to 11 nucleotides from one

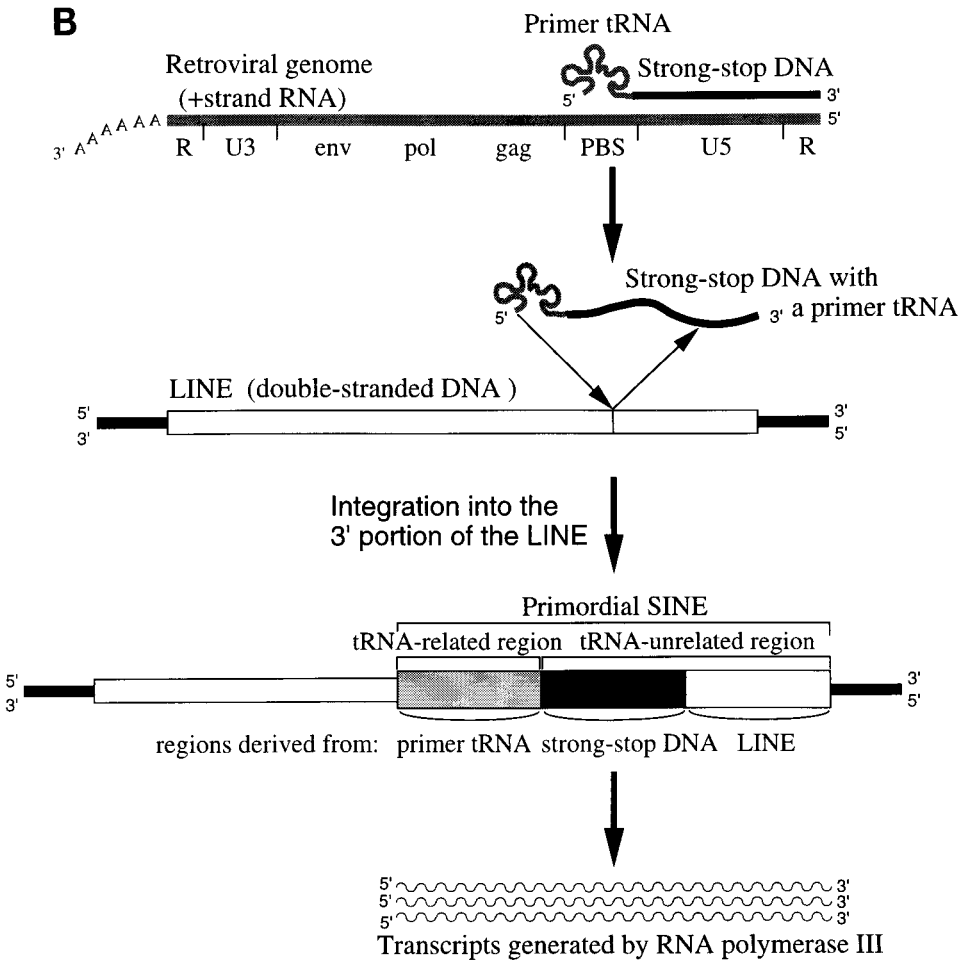

FIG. 9—*Continued*

another, can be found in the tRNA-unrelated regions of these five SINEs, suggesting a common evolutionary origin for the tRNA-unrelated regions of five SINEs and/or a certain function for this region. To our surprise, we found similar sequences in the sequences complementary to the U5 regions of several retroviruses that use $tRNA^{Lys}$ as a primer for reverse transcription. On the basis of these various observations, we proposed that a strong-stop DNA with a primer $tRNA^{Lys}$ was a primordial SINE during evolution (19).

We are now in a position to propose a more detailed possible scenario for the initial generation of SINEs, in which SINEs might have been generated by recombination between a strong-stop DNA with a primer tRNA and the DNA of a LINE. In the case of the tortoise Pol III/SINE and the salmon *Sma*I family, recombination is presumed to have occurred at a site within the two conserved motifs mentioned above (Fig. 8B). This observation suggests that some preference might exist for the choice of recombination sites and that the two conserved motifs might have been involved in such selection. It is now necessary to characterize more SINEs and their corresponding LINEs to reveal the nature of this preference.

The tortoise Pol III/SINE, consisting of the $tRNA^{Lys}$-related region plus the left half of the tRNA-unrelated region and the right half of the tRNA-unrelated region that is homologous to the 3′ end of the CR1-like LINE, is considered to have emerged in a common ancestor of cryptodires after the divergence of side-necked turtles (see Results). It might be argued that at that time, a functional SINE, in place of the strong-stop DNA plus $tRNA^{Lys}$, was inserted into a functional CR1-like LINE, thereby generating the composite structure of the tortoise Pol III/SINE family. Although we cannot rigorously exclude this possibility, we prefer our strong-stop DNA insertion model for the following reasons. First, if a functional SINE were to have been inserted into a functional LINE in the four cases described above, a simple repeated sequence, such as an AT-rich sequence and $(TTG)_n$, which is characteristic for the 3′ ends of tRNA-derived SINEs, should be present at the end of this putative functional SINE. Such sequences were not found in any cases examined. Second, if the tortoise Pol III/SINE had been generated by insertion of a shorter functional SINE into the CR1, this SINE should have arisen in the turtle genome before the emergence of the tortoise Pol III/SINE. However, we failed to detect any signals with side-necked turtle DNA when we performed dot blot analysis with the 5′ half of the tortoise Pol III/SINE as the probe (probe 2 in Fig. 2). Therefore, the tortoise Pol III/SINE seems likely to have appeared in the genome only after it had been combined with the 3′ end of the CR1-like LINE. These results suggest that such a shorter functional SINE might not have existed during evolution.

Figure 9B illustrates our most recent model for the initial generation of tRNA-derived SINEs during evolution.

The tortoise Pol III/SINE might be the direct descendant of a primordial SINE, generated by recombination between a strong-stop DNA with a primer $tRNA^{Lys}$ and a member of the CR1-like LINE family. It seems plausible that the tortoise Pol III/SINE might be the direct descendant of a primordial SINE, generated by recombination between a strong-stop DNA with a primer $tRNA^{Lys}$ and a member of the CR1-like LINE family. All of the members of the $tRNA^{Lys}$-related SINE superfamily are not, however, necessarily the direct descendants of primordial SINEs. It is possible that, after members of a primordial SINE had been generated, the 3′-tail region of a member might have been replaced by the 3′-tail region of a member of another LINE family, and this newly formed element might have generated a new family of SINEs. The *Hpa*I family and the *Ava*III family in salmonids might be examples of such a phenomenon. We suggest that the 3′ tail of the *Hpa*I family might have been derived from the RSg-1 LINE in salmonids. In the *Ava*III family, this 3′ tail was exactly replaced by another sequence with a different origin (12). In the future, we plan to accumulate data on pairs of SINEs that have a common head and different tails and pairs of SINEs that have different heads and a common tail.

It has been suggested that LINEs belong to the oldest group of retroelements and that LTR retrotransposons emerged after the acquisition of LTRs by a LINE (3, 38). The genuine retroviruses then emerged as a consequence of acquisition of *env* genes by LTR retrotransposons (see the introduction). The present study provides a possible mechanism for the creation of a novel retroelement by the combination of different kinds of retroelements, namely, LTR-type retrotransposons (retroviruses) and LINEs, that were already present into a single unit. Our results open a new perspective for a better understanding of the evolution of the genomes of higher eukaryotes, which appear to have been endowed with considerable structural fluidity and the potential for genetic variability by the presence of various LINEs and SINEs.

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