

Replication Origin of Mitochondrial DNA in Insects

Shigeru Saito,¹ Koichiro Tamura and Tadashi Aotsuka

Department of Biological Sciences, Tokyo Metropolitan University, Hachioji, Tokyo 192-0397, Japan

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ABSTRACT

The precise position of the replication origin (O_R) of mtDNA was determined for insect species belonging to four different orders (four species of *Drosophila*, *Bombyx mori*, *Tribolium castaneum*, and *Locusta migratoria*, which belong to Diptera, Lepidoptera, Coleoptera, and Orthoptera, respectively). Since the free 5' ends of the DNA strands of mtDNA are interpreted as the O_R , their positions were mapped at 1-nucleotide resolution within the A + T-rich region by using the ligation-mediated PCR method. In all species examined, the free 5' ends were found within a very narrow range of several nucleotides in the A + T-rich region. For four species of *Drosophila*, *B. mori*, and *T. castaneum*, which belong to holometabolous insects, although the O_R 's were located at different positions, they were located immediately downstream of a series of thymine nucleotides, the so-called T-stretch. These results strongly indicate that the T-stretch is involved in the recognition of the O_R of mtDNA at least among holometabolous insects. For *L. migratoria* (hemimetabolous insect), on the other hand, none of the long stretches of T's was found in the upstream portion of the O_R , suggesting that the regulatory sequences involved in the replication initiation process have changed through insect evolution.

METAZOAN mitochondrial DNA (mtDNA) is a closed-circular, double-stranded molecule, ranging in size from 15 to 20 kb (WOLSTENHOLME 1992). It contains a distinct replication origin (O_R) on each of the DNA strands. The position of the O_R and mode of replication have been examined in detail in mammalian mtDNA. The round of replication begins from the O_R of the H-strand and DNA synthesis proceeds unidirectionally. When the synthesis of the H-strand reaches two-thirds of the genome, the synthesis of the L-strand is initiated from the O_R of the L-strand located two-thirds of the genomic distance away from the O_R of the H-strand. Synthesis of the L-strand also proceeds unidirectionally (SHADEL and CLAYTON 1997; TAANMAN 1999).

Initiation of mtDNA replication is controlled by the interaction between nuclear-encoded proteins and regulatory sequences existing on the mtDNA (CLAYTON 1982; SHADEL and CLAYTON 1997; TAANMAN 1999). Several regulatory sequences have been identified in the noncoding region of the vertebrate mtDNA. These are located around the O_R and are widely conserved among vertebrate species. Immediately upstream of the O_R of the H-strand, conserved sequence blocks (CSBs) are present and are suggested to be responsible for the ini-

tiation of the H-strand replication (TAPPER and CLAYTON 1981; CHANG and CLAYTON 1985; CHANG *et al.* 1985; CAIRNS and BOGENHAGEN 1986; KING and LOW 1987; KANG *et al.* 1997). CSBs are thought to be involved in generating the 3' ends of the RNA primers, which are required for the DNA synthesis of the H-strand (SHADEL and CLAYTON 1997; TAANMAN 1999). Around the O_R of the L-strand, the sequence that could form a stem-loop configuration is conserved among vertebrate species and is also suggested to be required for the initiation of replication (MARTENS and CLAYTON 1979; TAPPER and CLAYTON 1981; HIXSON *et al.* 1986). *In vitro* replication studies have suggested that this structure serves as the recognition structure for mtDNA primase, which provides a short RNA primer for L-strand synthesis, and DNA synthesis is initiated near the base of the stem-loop structure utilizing the 3' ends of the RNA primer (HIXSON *et al.* 1986).

In invertebrate species, *Drosophila*, a member of the protostome species, is the only organism in which the position of the O_R and mode of replication have been examined. In several *Drosophila* species, replicative intermediates have been observed by electron microscopy (GODDARD and WOLSTENHOLME 1978, 1980). In *Drosophila* mtDNA, the leading strand and lagging strand are termed minor and major coding strands according to the relative numbers of the gene encoded on the respective DNA strand (GARESSE 1988). The O_R for the minor coding strand (O_N) is located somewhere in the middle portion of the large noncoding region called the "A + T-rich region" due to its extremely high adenine and thymine contents. However, observation using

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AB198740, AB198741, and AB212084.

¹Corresponding author: Laboratory of Bioscience, Department of Welfare Engineering, Faculty of Engineering, Iwate University, 4-3-5, Ueda, Morioka, Iwate 020-8551, Japan. E-mail: shigeru@iwate-u.ac.jp

electron microscopy did not allow determination of the precise location of the O_N . Synthesis of the minor coding strand proceeds unidirectionally, and the major coding strand synthesis begins after 97% of the minor coding strand synthesis is completed (GODDARD and WOLSTENHOLME 1978, 1980). The position of the O_R for the major coding strand (O_J) is unknown.

Since the A + T-rich region contains the O_N , this region is thought to be involved in controlling of mtDNA replication in *Drosophila* (WOLSTENHOLME 1992). However, the regulatory sequences involved in replication initiation have not been identified; thus, the role of the control region in the replication initiation process of invertebrate mtDNA is poorly understood. Comparative sequence analyses of the A + T-rich region have been carried out, and the conserved sequences among *Drosophila* species were also found (CLARY and WOLSTENHOLME 1987; MONFORTE *et al.* 1993; LEWIS *et al.* 1994; INOHIRA *et al.* 1997; BREHM *et al.* 2001; TSUJINO *et al.* 2002). These conserved sequences are proposed to play a role in regulation of mtDNA replication. However, they differ from conserved regulatory sequences reported in the vertebrate mtDNA. These observations indicate that regulatory sequences of mtDNA are different in invertebrate and vertebrate species and therefore suggest that the regulatory systems have changed through the evolution of animals.

Regulatory sequences are expected to exist near the O_R as in the case of vertebrate mtDNA (MARTENS and CLAYTON 1979; TAPPER and CLAYTON 1981; WALBERG and CLAYTON 1981). Thus, the O_R mapping on the nucleotide level is a necessary step for identifying potential regulatory sequences in the replication initiation process. In this study, we first attempted to determine the position of the O_R for four *Drosophila* species (*Drosophila yakuba*, *D. obscura*, *D. albomicans*, and *D. virilis*). Since replication of *Drosophila* mtDNA proceeds unidirectionally from the O_R (GODDARD and WOLSTENHOLME 1978, 1980), nascent DNA strands possess the free 5' ends at the O_R . Therefore we determined the position of the free 5' ends of the DNA strand at 1-nucleotide resolution within the A + T-rich region for both strands, using the ligation-mediated PCR (LMPCR) method (MUELLER *et al.* 1992; KANG *et al.* 1997). Then, the nucleotide sequences around the O_R were compared among the four *Drosophila* species to identify potential regulatory sequences that may be involved in the replication initiation process. The relationships between the O_R and conserved structure previously reported in the A + T-rich region of mtDNA in *Drosophila* are also discussed (CLARY and WOLSTENHOLME 1987; MONFORTE *et al.* 1993; LEWIS *et al.* 1994; INOHIRA *et al.* 1997; BREHM *et al.* 2001; TSUJINO *et al.* 2002). In addition, we also examined the position of the O_R for three other insect species belonging to different orders: silkworm, *Bombyx mori* (Lepidoptera); red flour beetle, *Triborium castaneum* (Coleoptera); and migratory locust, *Locusta*

migratoria (Orthoptera). Including *Drosophila*, which belongs to Diptera, the flanking nucleotide sequences of the O_R were compared among species belonging to the four different orders that are phylogenetically distantly related to one another. Our data provide new insights into the evolutionary changes of the regulatory sequences involved in replication of insect mtDNA.

MATERIALS AND METHODS

Insect species: *D. yakuba* and *D. obscura* belong to the subgenus *Sophophora* and are members of the *D. melanogaster* and the *D. obscura* species groups, respectively. *D. albomicans* and *D. virilis* belong to the subgenus *Drosophila* and are members of the *D. immigrans* and the *D. virilis* species groups, respectively. The isofemale line of *D. yakuba* was derived from Nairobi (Kenya), *D. obscura* was from Tuebingen (Germany), and *D. albomicans* was from San-hu-tang (Taiwan). The strain of *D. virilis* is an inbred strain. Silkworm *B. mori*, Red flour beetle *T. castaneum*, and migratory locust *L. migratoria* belong to Lepidoptera, Coleoptera, and Orthoptera, respectively. The *B. mori* strain is kinshu × showa, which was purchased from Ueda Sanshu. The *T. castaneum* strain has been maintained in a laboratory for >25 years. *L. migratoria* was collected from Tokyo and Tateyama, Japan.

Isolation of mtDNA: Mitochondrial DNA was extracted according to the method described by TAMURA and AOTSUKA (1988), omitting the alkaline lysis procedure. In the case of *Drosophila*, live adult flies (1.0 g) were homogenized in 40 ml of a chilled buffer containing 0.25 M sucrose, 10 mM EDTA, and 30 mM Tris-HCl (pH 7.5). The homogenate was centrifuged at $1000 \times g$ for 5 min at 4°, and the supernatant was retained. The centrifugation process was repeated three to four times. The resulting supernatant was centrifuged at $12,000 \times g$ for 10 min at 4°, and the pellet was resuspended in 4 ml of a buffer containing 0.15 M NaCl, 10 mM EDTA, and 10 mM Tris-HCl (pH 8.0). Then, 100 µl of 20% SDS was added to the mixture and incubated at room temperature for 30 min. DNA was extracted twice with phenol and once with phenol/chloroform and then precipitated with ethanol. The pellet was dried and dissolved in 30 µl of TE buffer containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. Then, DNA was treated with RNase A (20 µg/ml) at 37° for 30 min. In the case of *T. castaneum*, live adults were used, and for *B. mori* and *L. migratoria*, eggs from one female were used for mtDNA extraction.

Ligation-mediated PCR: LMPCR was performed according to previously described methods (MUELLER *et al.* 1992; KANG *et al.* 1997). The primers used in this study are shown in Figure 1. A unidirectional linker was prepared by hybridizing M13F20 (5'-GTTGTAACGACGGCCAGT-3') and M13FC (5'-ACTG GCCG-3'). Primer extension was performed in 30 µl of reaction mixture containing 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM MgCl₂, 0.001% gelatin, 200 µM of each dNTP, 0.3 pmol of primer 1, mtDNA (~15–80 ng), and 1.25 units of *Taq* DNA polymerase. The reaction conditions were 2 min at 95°, 30 min at 54°–64°, and 10 min at 72°. In the case of *Drosophila* species, the ND2-C primer (5'-GCTCTTAGTATTTCATCTAA ATG-3') was used as primer 1 for the major coding strand. The annealing temperature for primer extension was 54°. For the minor coding strand, the 12SBR primer (5'-AGCGACG GCGATGTGTACA-5') was used as primer 1 and the annealing temperature was 64°. In the case of *B. mori*, *T. castaneum*, and *L. migratoria*, the 12SBR2 primer (5'-GAAAGCGACGGG CAATATGT-3') was used as primer 1 for the minor coding strand. The annealing temperature for primer extension was 60°. After the primer extension reaction, 0.00625 units of KOD

polymerase (TOYOBO) was added to the mixture and incubated for 10 min at 68° to obtain the primer extension products with blunt ends. To ligate the unidirectional linker to the blunt end of the primer extension product, 43 µl of ligation mix was added to the mixture, which was then incubated at 16° for >12 hr. The ligation mix contained 52 mM Tris-HCl (pH 7.5), 14 mM MgCl₂, 35 mM dithiothreitol (DTT), 19 µg/ml bovine serum albumin (BSA), 100 pmol of unidirectional linker, and 350 units of T4 DNA ligase. After the ligation reaction, DNA was purified according to the method of BOOM *et al.* (1990). DNA was eluted in 20 µl of TE buffer.

To amplify the DNA, PCR was carried out in 30 µl of 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM MgCl₂, 0.001% gelatin, 200 µM of each dNTP, 5 pmol of the M13F20 primer, 5 pmol of primer 2, 0.5 µl of the purified primer extension product, and 1.88 units of *Taq* DNA polymerase. The reaction was carried out for 30 sec at 95°, for 1 min at 55°–65°, and for 1–2 min at 72° for 35 cycles, followed by an extension for 7 min at 72°. In the case of *Drosophila* species, the A + Tmet primer (5'-GGTATGAACCCAGTAGTAGCTTA-3') was used as primer 2 for the major coding strand. The annealing temperature was 55°. Using the PCR product obtained via the A + Tmet primer as a template, the second PCR was further carried out with 5-carboxyfluorescein-labeled A + Ttle primer (5'-GCATGATTACCCTATCAAG-3') as primer 3 to label the PCR products. The annealing temperature was 55°. For the minor coding strand, the 12SARacc2 primer (5'-CCGCGAYTGCTGGCACC AAT-3') for *Drosophila*, 12SBR for *B. mori*, and 12SCR (5'-ATAACCGCRACTGCTGGCAC-3') for *T. castaneum* and *L. migratoria* were used as primer 2. The annealing temperatures were 64° for *Drosophila*, 60° for *B. mori* and *T. castaneum*, and 65° for *L. migratoria*. Using the PCR product obtained via primer 2, the second PCR was further carried out using the following 5-carboxyfluorescein-labeled primers as primer 3 to label the PCR products: The A + Tvar-yak primer (5'-CTAAATCTGATAACTTATTTCCC-3') was used for *D. yakuba*, the A + Tvar-obs primer (5'-ATAAATTTTATAGAACAATTAACCTT-3') for *D. obscura*, the A + Tvar-alb primer (5'-AATTATTGTTCCCTTATAGAC-3') for *D. albomicans*, the A + Tvar-vir primer (5'-AAATTAATACCAATAACCCTA-3') for *D. virilis*, the 12SCR primer for *B. mori*, the A + Tcast primer (5'-GCTCAGAAAATAATTTCTCTGC-3') for *T. castaneum*, and the A + Tlmig primer (5'-AGAATAACTACTGCCCTCAGC-3') for *L. migratoria*. The annealing temperatures were 50° for *D. obscura* and *D. virilis*, 55° for *D. yakuba* and *D. albomicans*, 60° for *B. mori*, and 58° for *T. castaneum* and *L. migratoria*. After the labeling reaction, 0.00625 unit of KOD polymerase was added to the PCR reaction mixture and incubated for 10 min at 68° to obtain the PCR products with blunt ends.

Determination of the positions of the free 5' ends: The positions of the free 5' ends in all seven insect species were determined by the following method. The amplified PCR products obtained with the labeled primer were electrophoresed in a 5% acrylamide/6 M urea gel by using an ABI prism 377 sequencer (Perkin-Elmer, Norwalk, CT). A sequence ladder obtained with the same primer was applied in parallel with the PCR products to determine the positions of the free 5' ends. The sequence reaction was performed with the DYEnamic Direct cycle sequence kit (Amersham, Arlington Heights, IL), using the DNA fragment containing the A + T-rich region as a template. The minor coding strand in *D. obscura*, *D. albomicans*, *D. virilis*, *B. mori*, *T. castaneum*, and *L. migratoria* and the major coding strand in *D. albomicans* were also determined by the following method. The amplified PCR product was cloned into the pUC118 or pUC119 plasmid vector and sequenced (SAMBROOK and RUSSELL 2001).

Nucleotide sequence of the A + T-rich region: For sequencing of the entire A + T-rich region of *D. albomicans*, mtDNA was

digested with *Xba*I, and the DNA fragment containing the A + T-rich region was cloned into the plasmid vector pUC and sequenced (SAMBROOK and RUSSELL 2001). For *D. obscura*, the entire A + T-rich region was amplified by PCR with primers 5'-CTGCATGATTTACCCTAT-3' and 5'-GGAATTCTAAGCCAA AATAAACTT-3' and cloned into the pUC vector and sequenced. For *L. migratoria*, the DNA fragment containing the entire A + T-rich region and part of the 12S *rRNA* gene was amplified with the 12SBR and 5'-CAAGATAACCCTTTAATC AGGCA-3' primers, and the nucleotide sequence of this DNA fragment was determined by direct sequencing with the 12SBR and (5'-TAGGGTATCTAATCCAGT-3') primers. Sequence reactions were performed by using the ABI PRISM BigDye Terminator cycle sequencing kit (Perkin-Elmer) according to the supplier's instructions. Then nucleotide sequences were determined on an ABI PRISM 377 DNA sequencer (Perkin-Elmer). The nucleotide sequences determined in this study were deposited with the EMBL/GenBank Data Libraries under accession nos. AB198740, AB198741, and AB212084.

Nucleotide sequences of the A + T-rich region were obtained from GenBank for the following species: *D. yakuba* (NC001322), *D. virilis* (X05914), *D. subobscura* subL (AJ132900), *D. subobscura* subR (AJ132899), *D. madeirensis* (AJ132902), *D. guanche* (AJ132901), *D. teissieri* (X54011), *D. melanogaster* (U37541), *D. simulans* (AB003095, AB003096), *D. mauritiana* (AB003097, AB003098), *B. mori* (AB070264), and *T. castaneum* (AJ312413).

RESULTS

Positions of the free 5' ends of the minor and major coding strands in insect mtDNA: The positions of the free 5' ends of the minor coding strand were determined in *D. yakuba*, *D. obscura*, *D. albomicans*, and *D. virilis* using LMPCR. Figure 1 shows the direction of the replication and the location of the primers used for LMPCR. Primer extension was carried out with the 12SBR primer and mtDNA as a template. Then, the DNA was amplified by PCR with the 12SARacc2 primer. In each of the PCRs, the M13F20 primer was used as a linker primer. Only the nascent DNA strands initiated from the O_N in the A + T-rich region and passing beyond the 3' portion of the 12S *rRNA* gene were amplified by this primer set. DNA fragments of ~850, 900, 660, and 730 bp were amplified for *D. yakuba*, *D. obscura*, *D. albomicans*, and *D. virilis*, respectively. The results indicate that the free 5' ends were located in the middle portion of the A + T-rich region in all four species. Since the 12SARacc2 primer was relatively far from the free 5' ends (Figure 1), internal primers were used to determine the positions of the free 5' ends more accurately. Thus, a second PCR was carried out with the labeled A + Tvar primers. The resultant PCR product was resolved by a denaturing gel with a sequence ladder that was applied in parallel to determine the positions of the free 5' ends. Figure 2A shows the electrophoretogram and the schematic structure of the LMPCR product. The positions of the free 5' ends (indicated by an arrowhead) were 20 bp downstream of the sites where signals were observed (indicated by an arrow) because linker primers were attached to the 5' ends of the LMPCR products. The free 5' ends are indicated above the nucleotide

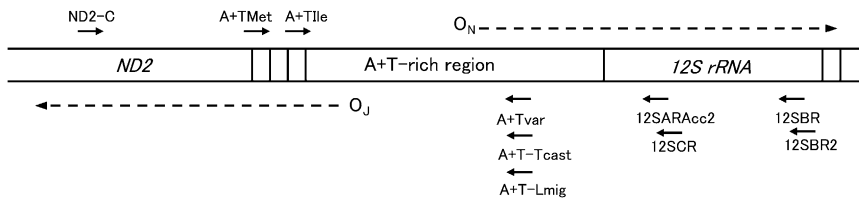


FIGURE 1.—The locations of primers on the minor coding strand (top strand) and the major coding strand (bottom strand) of insect mtDNA around the O_R . Arrows indicate primers with their names. The direction of replication from the O_R of the minor (O_N) and major (O_J) coding strands is indicated by arrows with dashed lines. *ND2* is the gene of *NADH dehydrogenase subunit 2*.

sequence in Figure 2B. The free 5' ends were designated as minor or major ends according to the relative intensity of the bands. The free 5' ends were mapped at three sites located adjacently for all four species examined. The major free 5' ends were located 441 bp from the *tRNA^{Leu}* gene for *D. yakuba*, 470 and 471 bp for *D. obscura*, 437 bp for *D. albomicans*, and 511 bp for *D. virilis*

in the middle portion of the A + T-rich region. The free 5' ends were located at similar positions relative to the *tRNA^{Leu}* gene for *D. yakuba* and *D. albomicans*; however, for *D. obscura* and *D. virilis* they were located ~30 and 70 bp downstream of the former two species, respectively.

We further determined the positions of the free 5' ends of the minor coding strand for three insect species

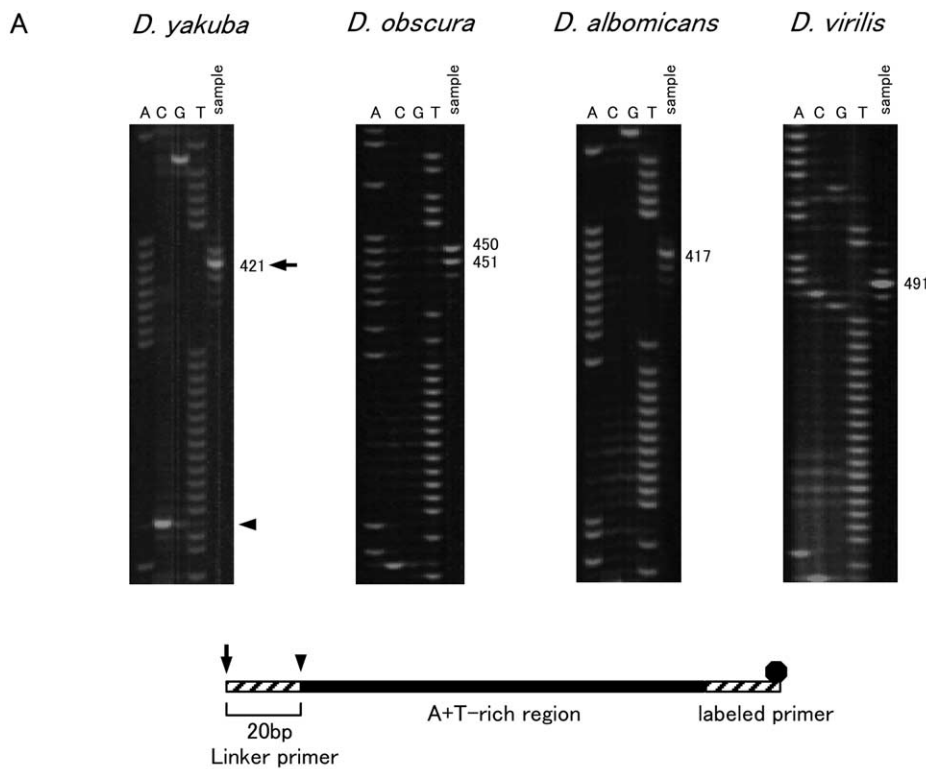
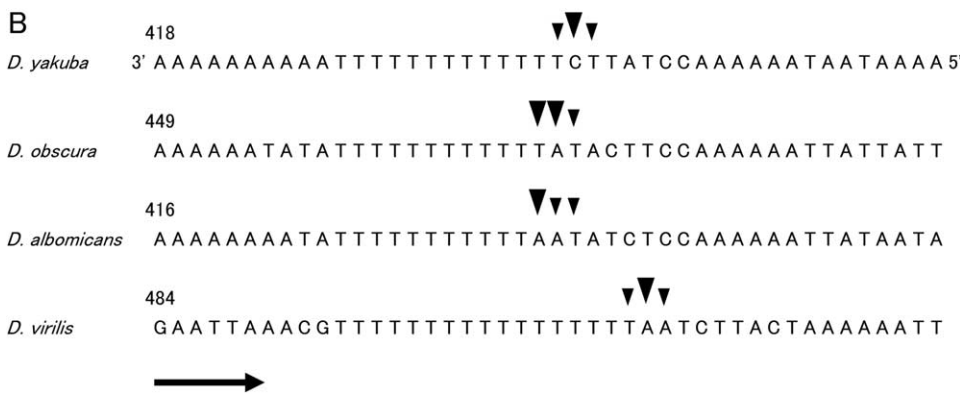


FIGURE 2.—Mapping of the free 5' ends for determining the position of the O_N of *Drosophila* mtDNA. (A) The nascent DNA strands of the minor coding strand were amplified with 5-carboxyfluorescein-labeled primer, and amplified product was resolved in a 5% acrylamide/6 M urea gel (sample lane). Sequence ladders were applied in parallel (lanes A, C, G, and T). The nucleotide positions with major signals are indicated on the right. The schematic structure of LMP-PCR product is shown below. Primers, the portion of the A + T-rich region, and the fluorescent dye are shown as a hatched box, solid box, and solid octagon, respectively. Arrows and arrowheads indicate the 5' ends of the PCR products and free 5' ends of the nascent DNA strands. (B) Location of the free 5' ends marking the O_N . The site of the nucleotide with the free 5' end is corrected by 20 bases for the length of the linker from the site where the signal was observed in A. Large arrowheads indicate the sites where major signals were observed and small arrowheads show the sites where minor signals were observed. Arrows indicate the direction of replication. The sequences of the template strands are in the middle portion of the A + T-rich region. The sequences shown for *D. yakuba* and *D. virilis* are from CLARY and WOLSTENHOLME (1985, 1987), respectively. The numbering above the sequence begins with the nucleotide that is next to the *tRNA^{Leu}* gene and proceeds through the A + T-rich region. Nucleotide position 1 for the sequence of *D. yakuba* corresponds to position 16019 of the sequence for the same species according to CLARY and WOLSTENHOLME (1985). The actual length of the T-stretch in the major coding strand for the strains in this study is 18 bp for *D. virilis*.



tion 1 for the sequence of *D. yakuba* corresponds to position 16019 of the sequence for the same species according to CLARY and WOLSTENHOLME (1985). The actual length of the T-stretch in the major coding strand for the strains in this study is 18 bp for *D. virilis*.

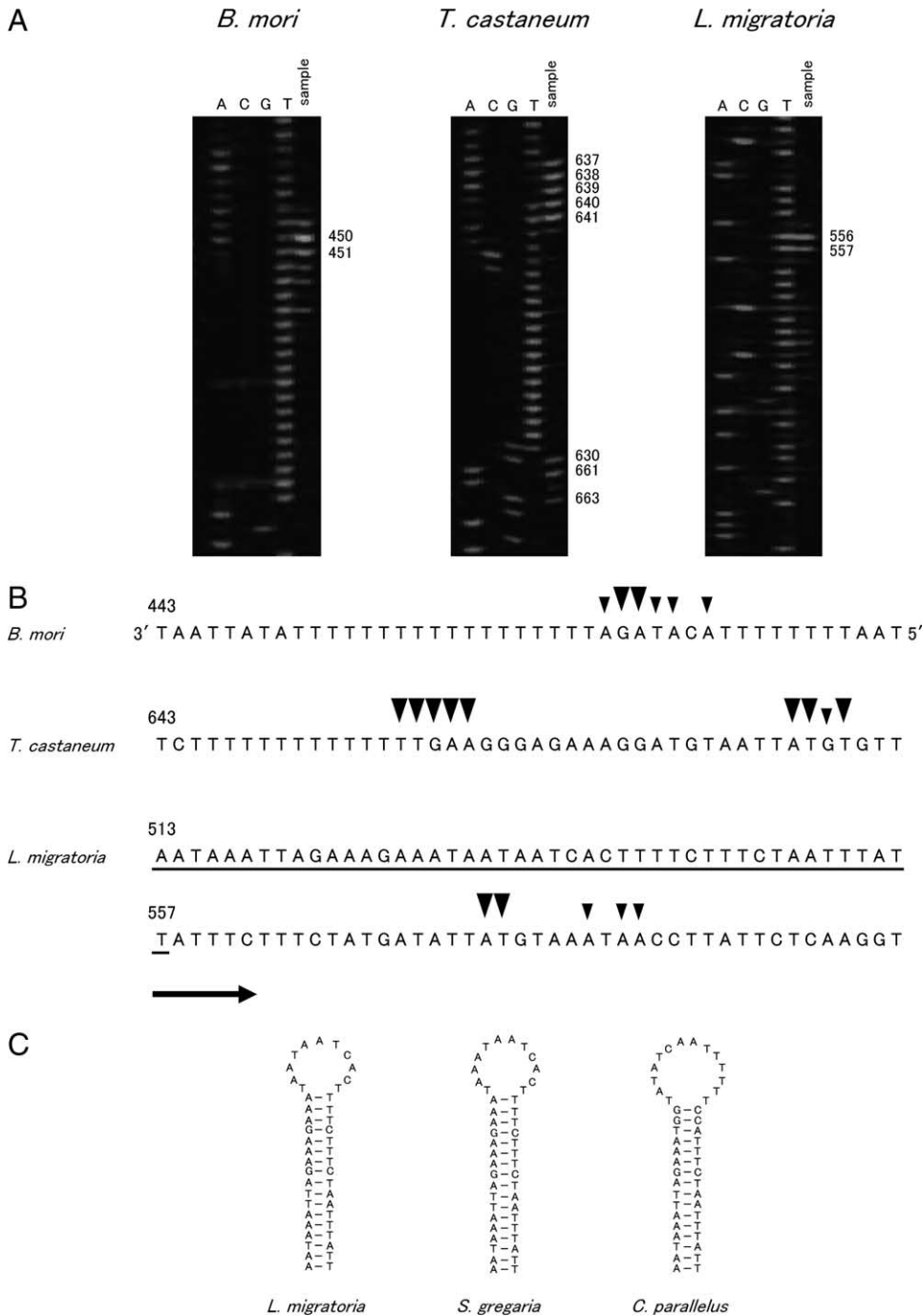


FIGURE 3.—Mapping of the free 5' ends for determining the position of the O_N of insect mtDNA. (A) The nascent DNA strands of the minor coding strand were amplified, and LMPCR product was resolved in a 5% acrylamide/6 M urea gel (sample lane) with the sequence ladders, which were applied in parallel (Lanes A, C, G, and T). (B) Location of the free 5' ends marking the O_N . The sequences of the template strands are the portion that is next to the 12S rRNA gene for *B. mori* and the middle portion for *T. castaneum* and *L. migratoria* in the A + T-rich region. The sequences shown for *B. mori* and *T. castaneum* are from YUKUHIRO *et al.* (2002) and FRIEDRICH and MUQIM (2003), respectively. See Figure 2 for an explanation of symbols. (C) Possible stem-loop structures formed immediately upstream of the O_N of *L. migratoria* mtDNA. The nucleotide sequence of *L. migratoria*, which potentially forms the stem-loop structure, is underlined in B. The possible stem-loop structures of *S. gregaria* and *C. parallelus* are from ZHANG *et al.* (1995).

belonging to different orders. Primer extension was carried out with the 12SBR2 primer (Figure 1). Then, DNA was amplified with the 12SBR primer for *B. mori* and with the 12SCR primer for *T. castaneum* and *L. migratoria*. DNA fragments of ~760, 800, and 860 bp were obtained for *B. mori*, *T. castaneum*, and *L. migratoria*, respectively. To determine the positions of the free 5' ends, sequential PCR was repeated using these PCR products as a template with the labeled 12SCR, A + T-Tcast, and A + T-Lmig primers for *B. mori*, *T. castaneum*, and *L. migratoria*, respectively. Figure 3 shows the electrophoretogram (Figure 3A) and the sites where the

free 5' ends were detected (Figure 3B). In each of the species, several signals were detected at adjacent sites. The major free 5' ends were mapped at 470 and 471 bp from the *tRNA^{Met}* gene for *B. mori* (in the case of *B. mori*, the A + T-rich region is flanked by the *tRNA^{Met}* gene instead of by the *tRNA^{Ile}* gene); at 657–661, 680, 681, and 683 bp from the *tRNA^{Ile}* gene for *T. castaneum*; and at 576 and 577 bp from the *tRNA^{Ile}* gene for *L. migratoria* within the A + T-rich region. The positions of the free 5' ends in the A + T-rich region are also indicated in Figure 4. The free 5' ends were located near the 12S rRNA gene for *B. mori* and at the middle portion of the A + T-rich

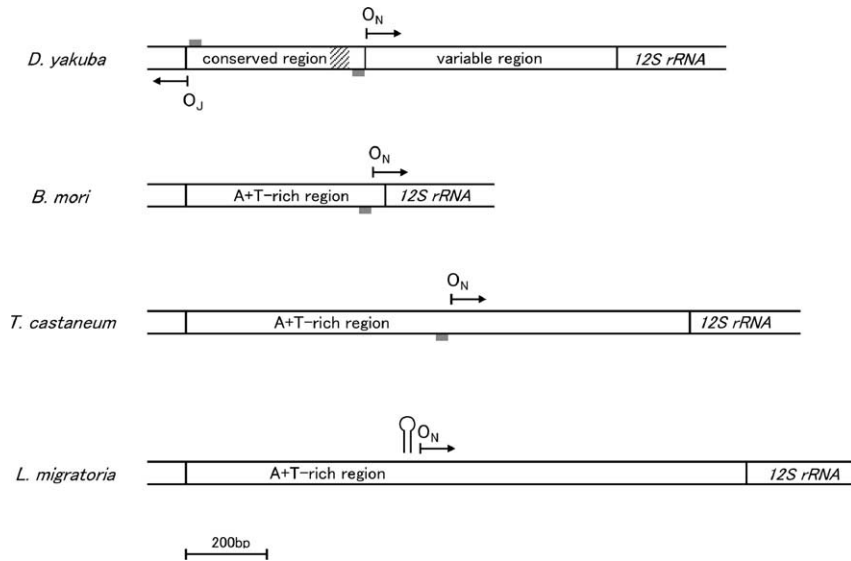


FIGURE 4.—The positions of the O_R of mtDNA for insect species belonging to four different orders. The directions of replication from the O_N and the O_J are indicated by arrows. Shaded boxes denote the T-stretch. A hatched box indicates the 50-bp portion highly conserved among *Drosophila* species in the A + T-rich region. The possible stem-loop structure is indicated immediately upstream of the O_N of *L. migratoria*.

region for four species of *Drosophila*, *T. castaneum*, and *L. migratoria*. Therefore, the results of this study revealed that the O_N is located within the A + T-rich region for insect species belonging to four different orders, Diptera, Lepidoptera, Coleoptera, and Orthoptera.

The positions of the free 5' ends of the major coding strand in insect mtDNA have not previously been mapped. The positions of the free 5' ends were determined in *D. yakuba*, *D. obscura*, *D. albomicans*, and *D. virilis*. Under the assumption that replication is initiated within the A + T-rich region (Figure 1), we carried out primer extension with the ND2-C primer. The DNA was then amplified with the A + TMet primer. Using this PCR product as a template, sequential PCR was repeated with labeled A + T11e primer. Figure 5 shows the electrophoretogram (A) and the sites where the free 5' ends were detected (B). In each of the species, several signals were detected at adjacent sites. The major free 5' ends were located 18 and 19 bp from the *tRNA^{Leu}* gene for *D. yakuba*; 2, 18, and 19 bp for *D. obscura*; 20 and 21 bp for *D. albomicans*; and 19 bp for *D. virilis*. The free 5' ends were near the *tRNA^{Leu}* gene (Figure 4) and were located at similar positions in all four species.

The positions of the free 5' ends were also examined by sequencing the LMPCR products for the minor coding strand in *D. obscura*, *D. albomicans*, *D. virilis*, *B. mori*, *T. castaneum*, and *L. migratoria* and for the major coding strand in *D. albomicans*. We cloned and sequenced the DNA fragments obtained by LMPCR and confirmed that the sites where the free 5' ends were mapped corresponded to those detected using the labeled primer (data not shown).

Conservation of the T-stretch immediately upstream of the free 5' ends of insect mtDNA: A consecutive group of thymine nucleotides, the so called "T-stretch," is located immediately upstream of the sites where the free 5' ends were found on the template strand in four *Drosophila* species examined (Figures 2B and 5B). The T-stretch is known to be conserved near the *tRNA^{Leu}* gene

in the minor coding strand and at the center in the major coding strand of the A + T-rich region in all *Drosophila* species so far examined, although the position of the T-stretch in the major coding strand is not always confined to a narrow area (CLARY and WOLSTENHOLME 1985, 1987; MONNEROT *et al.* 1990; MONFORTE *et al.* 1993; LEWIS *et al.* 1994; INOHIRA *et al.* 1997; BREHM *et al.* 2001). Figure 6 shows the nucleotide sequences around the T-stretch for several *Drosophila* species that have been sequenced thus far. The nucleotide sequences are aligned with the T-stretches, and the nucleotides with boxes are the sites where the free 5' ends were mapped for the four species in this study. The long T-stretch was also found immediately upstream of the O_N for *B. mori* (18 bp in size) and *T. castaneum* (14 bp); however, none of the long stretches of T's was found in the upstream portion of the O_N for *L. migratoria* (Figure 3B). The flanking nucleotide sequences of the O_N were compared for four species of *Drosophila*, *B. mori*, *T. castaneum*, and *L. migratoria*; however, the portion excluding the T-stretch was highly variable among species belonging to different orders so that the nucleotide sequences were not reliably aligned. Therefore, none of the portions that are conserved among species belonging to different orders was found except for the T-stretch.

The length of the T-stretch varied among and within the species compared. The shortest T-stretch was 11 nucleotides on the major coding strand of *D. albomicans* and the longest was 25 nucleotides on the minor coding strand of *D. teisseri* (Figure 6). In *D. subobscura*, the length of the T-stretch on the major coding strand differed even within identical species, *i.e.*, 15 nucleotides in strain subL and 13 nucleotides in strain subR.

DISCUSSION

Replication origin of insect mtDNA: In this study, we reported for the first time the precise positions of

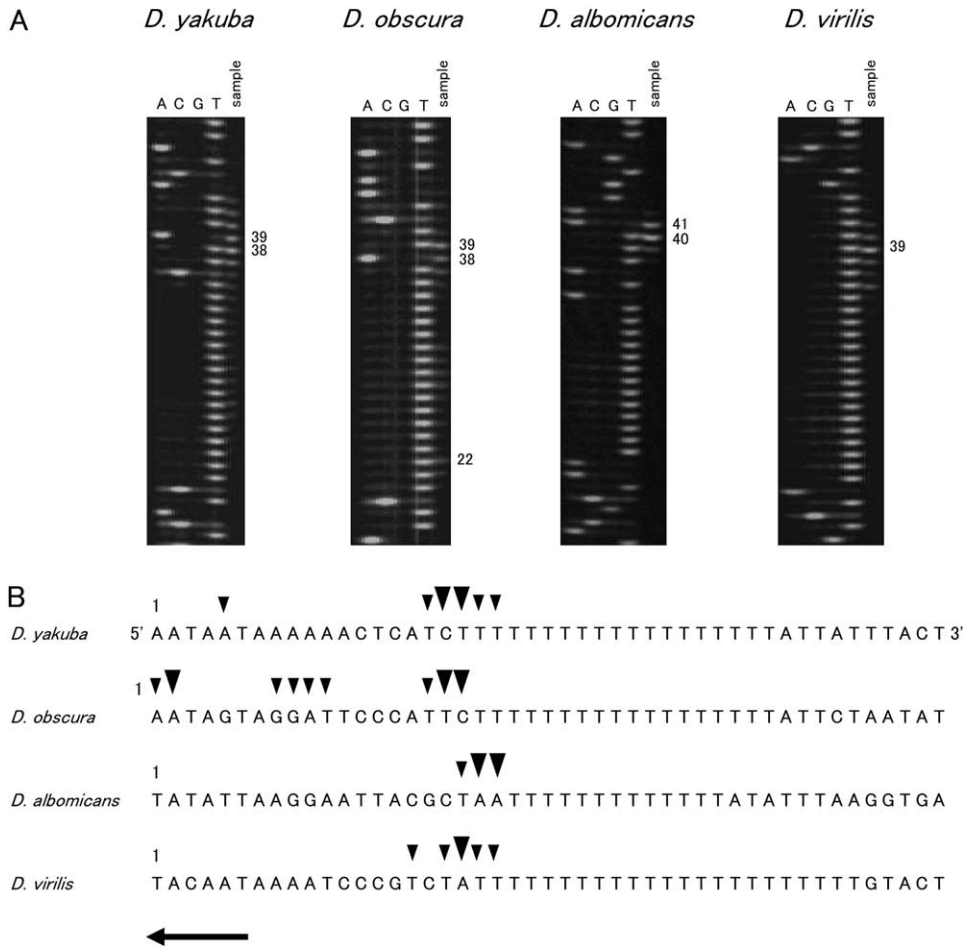


FIGURE 5.—Mapping of the free 5' ends for determining the position of the O_j of *Drosophila* mtDNA. (A) The nascent DNA strands of the major coding strand were amplified, and LMP-PCR product was resolved in a 5% acrylamide/6 M urea gel (sample lane) with the sequence ladders, which were applied in parallel (Lanes A, C, G, and T). (B) Location of the free 5' ends marking the O_j . The sequences of the template strands are the portion that is next to the $tRNA^{Leu}$ gene. The actual lengths of the T-stretch in the minor coding strands for the strains in this study are 17 and 24 nucleotides for *D. yakuba* and *D. virilis*, respectively. See Figure 2 for explanation of symbols.

the O_R 's of mtDNA by mapping the free 5' ends at I-nucleotide resolution for seven insect species that are phylogenetically distantly related (four species of *Drosophila*, *B. mori*, *T. castaneum*, and *L. migratoria*, which belong to Diptera, Lepidoptera, Coleoptera, and Orthoptera, respectively). In all species examined, the free 5' ends of the minor coding strands were found in a very narrow range of several nucleotides in the A + T-rich region, located at the middle portion for four *Drosophila* species, *T. castaneum*, and *L. migratoria* and near the 12S rRNA gene for *B. mori* (Figures 2B, 3B, and 4). *D. yakuba* and *D. virilis* were used in a previous mapping study using electron microscopy, and the free 5' ends were found in the middle of the A + T-rich region (GODDARD and WOLSTENHOLME 1980). Therefore, the results of the present study are consistent with the previous results.

The position of the O_j of *Drosophila* mtDNA has been unknown. However, we assumed that the O_j is also located within the A + T-rich region for the following reason. According to the strand-asynchronous, asymmetric model of vertebrate mtDNA, the replication of the L-strand is initiated when the synthesis of the H-strand passes beyond the L-strand origin, and the template strand for the L-strand replication becomes single stranded (CLAYTON 1982; SHADEL and CLAYTON 1997; TAANMAN 1999). In *Drosophila* mtDNA, the rep-

lication of the major coding strand initiates after 97% of the minor coding synthesis is completed (GODDARD and WOLSTENHOLME 1978, 1980). If the replication mode is similar between both *Drosophila* and vertebrate mtDNAs, the O_j must be located 97% of the genomic distance away from the O_N , that is, within the A + T-rich region. As expected, in this study, the free 5' ends were found in the A + T-rich region near the $tRNA^{Leu}$ gene (Figures 4 and 6), exactly 97% of the genomic distance away from the O_N .

In animal mtDNA, a strand-asynchronous, asymmetric model of replication has been widely accepted (CLAYTON 1982; SHADEL and CLAYTON 1997; TAANMAN 1999). However, recently in human and mouse mtDNA, a symmetrical model of replication has been proposed (HOLT *et al.* 2000; BOWMAKER *et al.* 2003). A symmetrical model differs greatly from a strand-asynchronous, asymmetric model in respect to the position of the O_R and the direction of replication. In a symmetrical model of replication, the O_R is located outside of the large non-coding region, the so-called D-loop region, and replication proceeds bidirectionally from the O_R . Therefore, in mammalian mtDNA, controversy exists regarding the mode of replication. On the other hand, in the case of *Drosophila*, only a strand-asynchronous, asymmetric mode has been reported to date (GODDARD and WOLSTENHOLME

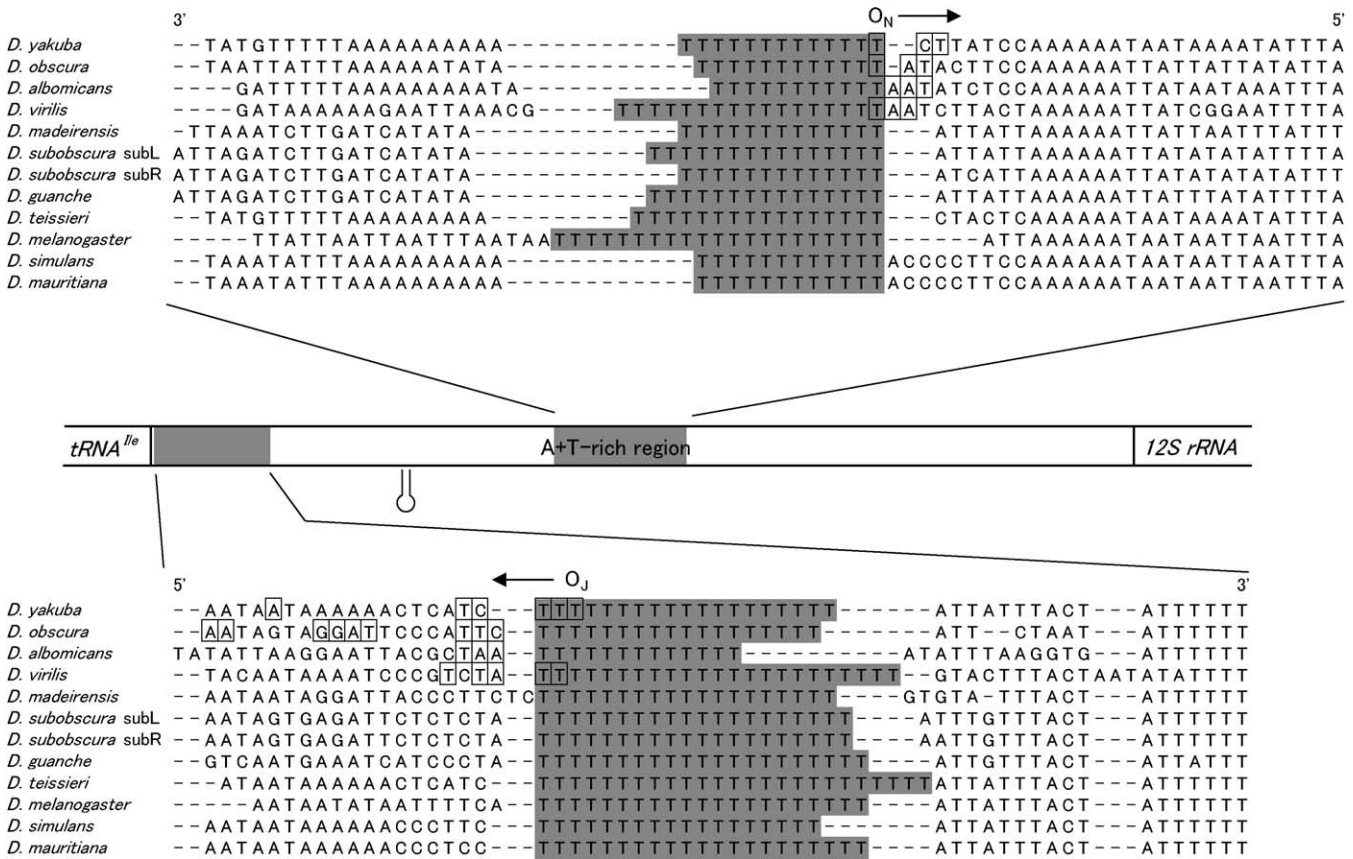


FIGURE 6.—The nucleotide sequences around the T-stretches and the positions of the O_R of *Drosophila* mtDNA. The nucleotide sequences for several *Drosophila* species that were determined thus far were aligned with the T-stretches (shaded areas) on the major coding (top) and the minor coding (bottom) strands, respectively. The sites where the free 5' ends were mapped for four *Drosophila* species in this study are indicated by boxes. The direction of replication is indicated by an arrow. The stem-loop structure previously proposed is also shown (CLARY and WOLSTENHOLME 1987; MONFORTE *et al.* 1993).

1978, 1980). The O_R mapping in the present study also supported the finding that *Drosophila* mtDNA replicates with a strand-asynchronous, asymmetric mode.

Relationship between the O_R and conserved sequences of the A + T-rich region of *Drosophila* mtDNA: Sequence analyses of the A + T-rich region of *Drosophila* mtDNA thus far showed that the A + T-rich region is divided into two parts according to the extent of sequence conservation (CLARY and WOLSTENHOLME 1987; MONFORTE *et al.* 1993; LEWIS *et al.* 1994). Half of the A + T-rich region (~430 bp) flanked by the *tRNA^{Ile}* gene is conserved among species and termed the conserved region, while the remaining part flanked by the *12S rRNA* gene is highly variable and termed the variable region (MONFORTE *et al.* 1993). The O_R mapping in the present study showed that the O_R 's are located near both ends of the conserved region of the A + T-rich region (Figure 4). With respect to the O_N , an ~50-bp portion (corresponding to position 357–411 in the *D. yakuba* sequence) located upstream of the T-stretch in the major coding strand (which serves as the template strand for the replication of the minor coding strand) is the most conservative portion of the A + T-rich region (Figure 4; see also CLARY and WOLSTENHOLME 1987; MONFORTE *et al.* 1993;

INOHIRA *et al.* 1997; BREHM *et al.* 2001). The degree of conservation of this 50-bp portion is approximately the same as that of the *12S rRNA* gene of *Drosophila* mtDNA (MONFORTE *et al.* 1993), suggesting that this portion is subject to a strong functional constraint. The round of replication of *Drosophila* mtDNA begins from the O_N (GODDARD and WOLSTENHOLME 1978, 1980); therefore, the total replication rate of mtDNA is determined by synthesis of the minor coding strand. Taking this fact into account, an ~50-bp conserved portion may play some role in the regulation of replication initiation.

Within the conserved region of the A + T-rich region of *Drosophila*, two conserved structures were proposed to be involved in the replication initiation process. A possible stem-loop structure that is located in the A + T-rich region has been conserved among several *Drosophila* species and has been speculated to act as the O_J (CLARY and WOLSTENHOLME 1987; MONFORTE *et al.* 1993; LEWIS *et al.* 1994; INOHIRA *et al.* 1997; BREHM *et al.* 2001; TSUJINO *et al.* 2002). The precise mapping of the O_J of *Drosophila* mtDNA in the present study revealed that the O_J was located near the *tRNA^{Ile}* gene and was ~230–240 bp away from the previously proposed stem-loop structures (Figure 6). Therefore,

a direct involvement of this stem-loop structure in replication initiation of *Drosophila* mtDNA is not supported by the present results.

Another conserved structure proposed to serve as the O_R was the T-stretch (LEWIS *et al.* 1994). Lewis *et al.* analyzed the nucleotide sequence of the A + T-rich region of *D. melanogaster* mtDNA. The A + T-rich region of this species is ~4.6 kb long and is organized into two large arrays of tandemly repeated sequences, type I (corresponding to the variable region) and type II (corresponding to the conserved region) repeats. Type I repeats are tandemly repeated five times and located in half of the A + T-rich region adjacent to the *12S rRNA* gene, and type II repeats are tandemly repeated four times and once partially and located adjacent to the *tRNA^{Leu}* gene. If the recognition sequence for replication initiation is included in the repeated sequence, the O_R 's must also be situated tandemly in *D. melanogaster* mtDNA. However, the O_N has been found only at the central portion of the A + T-rich region by electron microscopy (GODDARD and WOLSTENHOLME 1978). The prominent T-stretch in the major coding strand is located at the central intervening portion between the type I and II repeats and is excluded from the repeated sequences. The T-stretch in the minor coding strand is located near the *tRNA^{Leu}* gene and is also excluded from the repeated sequence. Therefore, the T-stretch has been proposed to be involved in the replication initiation process (LEWIS *et al.* 1994). The precise mapping of the O_R of *Drosophila* mtDNA in the present study revealed that the T-stretches are located immediately upstream of the O_R of both strands in all four species (Figures 2B and 5B). Furthermore, the T-stretches of several other *Drosophila* species that are not used in the present mapping study are also located at the center in the major coding strand and near the *tRNA^{Leu}* gene in the minor coding strand (Figure 6; see also CLARY and WOLSTENHOLME 1985, 1987; MONNEROT *et al.* 1990; MONFORTE *et al.* 1993; LEWIS *et al.* 1994; INOHIRA *et al.* 1997; BREHM *et al.* 2001; TSUJINO *et al.* 2002). Therefore, the T-stretches are conserved at the positions where the O_R 's are located for four *Drosophila* species that are used in the present mapping study. This strongly suggests that the T-stretch is involved in the recognition of the O_R of *Drosophila* mtDNA.

Possible function of the T-stretch of *Drosophila* mtDNA: Thymine nucleotide stretches are also found at the O_R 's of other systems. They are located immediately upstream of the L-strand origin of the mammalian mtDNA (CLAYTON 1982), upstream of the O_R of colicin E1 (TOMIZAWA *et al.* 1977), and in the core origin of simian virus 40 (DEB *et al.* 1986). In the case of simian virus 40, mutational analysis showed that the consecutive thymine nucleotides play an essential role in replication initiation (DEB *et al.* 1986). It has been shown in *in vitro* experiments that the consecutive thymine nucleotides induce a bending of the DNA (KORNBERG and BAKER

1992; SINDEN 1994). In simian virus 40, DNA bending of the T-stretch was shown to be required for binding of a protein that is essential for the initiation of replication (SINDEN 1994). The T-stretch is thought to serve as a structural signal for the recognition of proteins involved in replication initiation. In *Drosophila*, the T-stretches most likely also play an important role as a recognition sequence in replication initiation of mtDNA.

In the case of mammalian mtDNA, the T-stretch is located in the loop portion of the possible stem-loop structure in human, bovine, and mouse mtDNA and varies in size from 6 to 11 bp (ANDERSON *et al.* 1982). In human mtDNA, initiation of DNA synthesis requires a DNA primase, responsible for generating short RNA molecules with 5' ends that map to the T-stretch. DNA synthesis initiates from the sites near the base of the stem of the secondary structure, utilizing the free 3' ends of primer RNA (HIXSON *et al.* 1986). In the case of *Drosophila*, a stem-loop structure conserved among *Drosophila* species was not predicted by a computer program such as MFOLD version 3.1 (ZUKER *et al.* 1999). Therefore, the existence of the stem-loop structure around the O_R remains ambiguous in *Drosophila* mtDNA. However, the fact that mammals and *Drosophila* share the T-stretch immediately upstream of the O_R suggests that the T-stretch of *Drosophila* mtDNA may be involved in primer RNA synthesis for replication initiation. Precise mapping of both the 5' and 3' ends of RNA may provide important clues for understanding the function of the T-stretch and the replication initiation process. Although studies examining the protein-DNA interaction in the A + T-rich region are essential for understanding the replication initiation process of *Drosophila* mtDNA, only a few studies have been performed (POTTER *et al.* 1980; PARDUE *et al.* 1984). The identification of potentially functional sequences, such as the T-stretch, may provide useful information for analyzing the DNA-protein interaction of the *Drosophila* A + T-rich region in future studies.

Evolution of the nucleotide sequence around the O_R of insect mtDNA: In this study, flanking nucleotide sequences of the O_N were compared among species belonging to four different insect orders to understand the evolutionary changes of the regulatory sequences involved in the replication initiation process of insect mtDNA. From phylogenetic analysis using the morphological and molecular data, Orthoptera (hemimetabolous insect) is thought to have diverged first from the other three insect orders. Then, the Coleoptera diverged relatively early in holometabolous insect evolution, and Lepidoptera and Diptera diverged most recently (CARMEAN *et al.* 1992; WHEELER *et al.* 2001). For *Drosophila* species (Diptera), *B. mori* (Lepidoptera), and *T. castaneum* (Coleoptera), the long T-stretch was located immediately upstream of the O_R , while for *L. migratoria* (Orthoptera), none of the long stretches of T's was found in the upstream portion of the O_R

TABLE 1

Length of T-stretch in the A + T-rich region of insect mtDNA

Order	Species	Length of T-stretch (bp)	
		Major coding	Minor coding
Holometabola			
Diptera	<i>Drosophila</i>	11–17 ^a	13–23 ^a
	<i>Ceratitis capitata</i>	21	21
	<i>Chrysomya chloropyga</i>	23	19
	<i>Cochliomyia hominivorax</i>	25	23
	<i>Anopheles quadrimaculatus</i>	17	9
	<i>Anopheles gambiae</i>	17	6
Lepidoptera	<i>Culex torrentium</i>	19	9
	<i>Bombyx mori</i>	18 ^a	11
	<i>Cydia pomonella</i>	11	6
Hymenoptera	<i>Pandemis heparana</i>	11	9
	<i>Apis mellifera</i>	15	7
Coleoptera	<i>Tribolium castaneum</i>	14 ^a	9
	<i>Pyrocoelia rufa</i>	12	8
	<i>Crioceris duodecimpunctata</i>	17	12
Hemimetabola			
Thysanoptera	<i>Thrips imagines</i>	20	10
Hemiptera	<i>Rhopalosiphum padi</i>	11	6
	<i>Triatoma dimidiata</i>	6	9
Phthiraptera	<i>Heterodoxus macropus</i>	7	6
Orthoptera	<i>Locusta migratoria</i>	4	8
Ametabola			
Thysanura	<i>Tricholepidion gertschi</i>	5	6
Collembola	<i>Gomphiocephalus hodgsoni</i>	9	5
	<i>Tetrodontophora bielanensis</i>	4	6

^a Ascertained the location of the T-stretch immediately upstream of the O_R.

(Figures 3 and 6). The former three species that belong to holometabolous insects shared the common feature immediately upstream of the O_R; therefore, the long T-stretch seems to be conserved in holometabolous insects. To verify this idea, we searched for the T-stretch within the A + T-rich region of insect mtDNA for the species in which nucleotide sequences have been so far determined, since the long T-stretches would be found in the A + T-rich region if they are essential for mtDNA replication initiation of a wide variety of holometabolous insect species. Table 1 shows the longest T-stretch found on each of the DNA strands within the A + T-rich region. The length of the T-stretch ranged from 4 to 25 bp for the species examined. The minimum length of the T-stretch that is indispensable for the replication initiation of mtDNA is not known, while the lengths of the T-stretches found immediately upstream of the O_R's were >10 bp (Table 1). Therefore, in this study, the T-stretch >10 bp was considered as participating in the replication initiation of insect mtDNA. In the major coding strand shown in Table 1, the T-stretch (11–25 bp) was found for all holometabolous and some species

of hemimetabolous insects examined. This observation also suggests that the T-stretch is conserved immediately upstream of the O_N at least among holometabolous insects. In the minor coding strand shown in Table 1, several species of holometabolous insects do not possess the long T-stretch, whereas all the species of the suborder Brachycera thus far examined possess the T-stretch, ranging in size from 13 to 23 bp. Therefore, immediately upstream of the O_J, the T-stretch may be conserved at least among the species of Brachycera.

On the other hand, several hemimetabolous and ametabolous insects species did not possess the long T-stretch within the A + T-rich region on the major coding strand (Table 1). We ascertained that none of the long T-stretches was found in the upstream portion of the O_N of *L. migratoria* mtDNA (Figure 3B). These results suggest that a sequence other than the T-stretch may be involved in the replication initiation process in the species that do not possess the long T-stretch in the A + T-rich region. It is worth noting that a potential stem-loop structure existed 20 bases upstream of the O_N of *L. migratoria*, which was also located at a similar position on mtDNA of desert locust *Schistocerca gregaria* and the meadow grasshopper *Chorthippus parallelus* (Figures 3 and 4) (ZHANG *et al.* 1995). This possible stem-loop structure is a favorable candidate that may be involved in replication initiation since its conformation is highly similar to that of the stem-loop structure located around the L-strand origin of vertebrate mtDNA. To examine whether or not a stem-loop structure is conserved immediately upstream of the O_R, the determination of the precise position of the O_R for additional species is necessary, especially for the species that do not possess the long T-stretch within the A + T-rich region.

In this study, we revealed that the nucleotide sequences upstream of the O_R of mtDNA are conserved among insect species that are phylogenetically distantly related, while a difference in flanking nucleotide sequence of the O_R of insect mtDNA was also found. Furthermore, no sequence with high similarity to those of conserved regulatory sequences among vertebrate species, such as CSBs, was found around the O_R of insect mtDNA, although the T-stretches are located immediately upstream of the O_R of insect mtDNA and of the L-strand origin of mammalian mtDNA. Therefore, the sequence properties of the flanking portion of the O_R are different between insect and vertebrate mtDNA; namely, the replication system of mtDNA has evolved through the changes in the regulatory sequences.

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