Volume 14 Number 16 1986 Nucleic Acids Research

Molecular mechanism of introduction of the hidden break into the 28S rRNA of insects: implication based on structural studies

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Received 12 June 1986; Revised and Accepted 29 July 1986

Abstract

We determined the structure around the gap region between 28Sα and 28Sβ rRNA of Bombyx mori, a lepidopteran insect, to know the introduction mechanism of the hidden break, an interruption of the phosphodiester bond specific to the 28S rRNA of protostomes. Sequence analysis and S1 nuclease mapping suggested that a stretch of 30 nucleotides is excised from the mid region of the 28S rRNA to generate the hidden break. The length of excluded stretch was very various among three insects so far studied. However, the gap responsible for the hidden break was located in a fixed position in the 28S rRNA irrespective of insect species. It was suggested that extremely AU-rich sequence including the specific UAAU tract forming a loop can be a signal for the introduction of the hidden break. The same signal seemed also involved in splitting the dipteran 5.8S rRNA.

Introduction

The cytoplasmic ribosome of eukaryotic cells contains four RNA molecules, 28S, 18S, 5.8S and 5S rRNA. The coding region of 28S rRNA (28S rDNA) ranges in size from about 4000b in some unicellular organisms to 5000b in human (1,2).

The 28S rRNA from insects and most other protostomes is characterized by its dissociation into two equally-sized subunits, α and β, under denaturing conditions (3). The two are hydrogen-bonded to each other at the regions close to the cleavage site, which is at a halfway point of the 28S rRNA. This specific break, which is called "hidden break", has been also found with the 23S rRNA of higher plant chloroplasts (4), but not with any 28S rRNA of deuterostomes (5).

Molecular mechanism of introduction of the hidden break into the 28S rRNA of insects has not been clarified as yet because of insufficiency of information on the structure of the 28S rRNA of this group.

Recently, two groups have revealed the primary structures of and around the gap of the 28S rDNA from two dipteran species, Drosophila melanogaster (6,7) and Sciara coprophila (7). According to their reports, nucleotides of 19b in Sciara and about 60b in Drosophila (7) are excised from the respective 28S rRNA to generate the hidden break. However, comparison between such closely related species could hardly provide enough information to know of the introduction mechanism of the hidden break. The present study on the gap structure responsible for the hidden break of Bombyx mori 28S rRNA, will provide an opportunity to be better informed with the structure and recognition signals essential to the RNA processing of this special kind.

Materials and Methods

A recombinant phage library of Bombyx mori genes was constructed by insertion of partial and complete EcoRI digests into the Charon4A vector as described previously (8). A genomic rDNA clone, λBmR11, not containing insertion sequence (Int^-), was previously subcloned into pBR322 by insertion of 4.2kb EcoRI-BamHI fragment. This clone, termed pBmR145, was used for structural analysis of the hidden break region. Total RNA was extracted from posterior silk glands of the 5th-instar larvae of Bombyx using guanidinium/cesium chloride method (9). (γ-^{32}P)ATP was synthesized using ^{32}P-orthophosphoric acid (NEN) after Walseth et al (10). All sequencing reactions were performed using the method of Maxam and Gilbert (11). The sequencing gels containing 7M urea were run in 1x TEB buffer. S1-Nuclease mapping experiments were performed basically as described by Berk and Sharp (12).

Results

As shown Fig. 1, pBmR145 carries the 5'-half of the 28S rDNA of Bombyx mori. From the published data of Manning et al. (13) for Bombyx rDNA organization and our partial sequence data for the Bombyx 28S rDNA (8), the half point of the molecule was positioned at about several hundred nucleotides upstream of the BamHI site of pBmR145 (Fig. 1). Since the two subunits, α and β, of the Bombyx 28S rRNA are equally-sized, the site of the hidden break

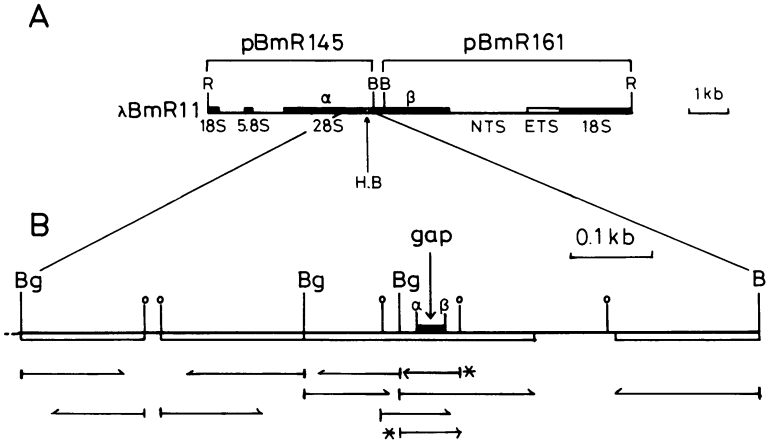

Fig. 1. Restriction map of Bombyx rRNA gene and the sequencing strategy for the gap and its flanking regions. A; the upper line shows one complete rDNA unit, which was inserted to Eco RI sites of Cha4A, with the restriction sites for EcoRI(R) and BamHI(B). The EcoRI-BamHI-generated fragments were subcloned in pBR322, respectively (pBmR145 and 161). NTS, ETS and H.B. denote the non-transcribed, external transcribed spacer and the hidden break region, respectively. B; the sequencing strategy for the BglII-BamHI region of pBmR145 is given. Arrows indicate the direction and the size of the sequenced DNA fragments. The region whose nucleotide sequence was determined (about 700 bp) was indicated as open-boxes under the line. The filed box represents the gap region between the 3'-end of 28Sα and the 5'-end of 28Sβ molecule. ↑, the restriction site of HinfI. ⊣⊢, single stranded probes for S1 nuclease mapping.

along the 28S rDNA should reside also somewhat upstream of the BamHI site of pBmR145.

To permit identifying the positions corresponding to the 3'-end of 28Sα RNA and the 5'-end of 28Sβ RNA, respectively, at the nucleotide level, nucleotide sequence extending over about 700bp flanking the gap region was determined using a strategy shown in Fig. 1B. Thus obtained rDNA sequence was shown in Fig. 2 in comparison with that of the corresponding region of Xenopus laevis rDNA (14). The sequence comparison suggested that both the 3'-end of the 28Sα and the 5'-end of the 28Sβ RNA lie within the region between the third BglII and fourth HinfI site indicated by arrows in Fig. 2. Each of these restriction sites was labeled for a size determination of the protected fragments in the S1-nuclease mapping.

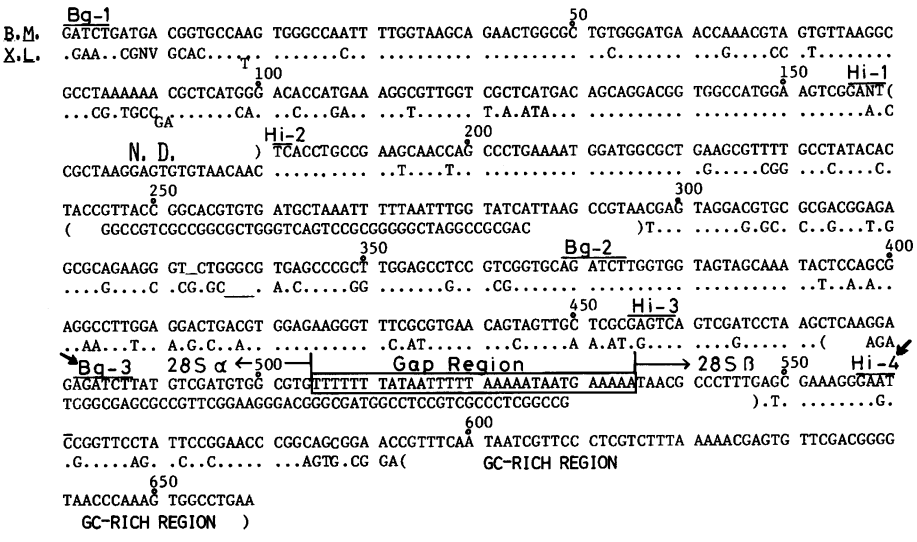

Fig. 2. Sequence of the gap and its flanking region of Bombyx 28S rDNA. The sequence of the RNA-like strand of about 700bp region between BglII and BamHI site (see Fig. 1), was shown in the upper line (B. M. represents Bombyx mori). The corresponding region of Xenopus (X. L.) 28S rDNA was aligned in the lower line (Ref.(14)). Dots indicate nucleotides found common in the Bombyx sequence. Bars present gaps introduced to maximize homology. The gap region of Bombyx mori was boxed. Bg and Hi represent the restriction sites for BglII and HinfI, respectively and numbered from the upstream region. The sequences of Xenopus rDNA in parentheses are not homologous to the corresponding regions of the Bombyx 28S rDNA.

Figure 3 shows the results of this size determination of the DNA fragments protected by Bombyx rRNA on sequencing gels, alongside the sequencing ladders of the same DNA fragment. To detect the 5'-end of 28Sβ rRNA, the 80bp BglII-HinfI fragment was labeled only at the 5'-end of the HinfI site. The rRNA-protected band (Fig. 3A lane 1 and 2) was determined to be 25 nucleotides long, which positioned the 5'-end of 28Sβ rRNA. To determine the 3'-end of 28Sα rRNA, the same BglII-HinfI fragment was labeled at the 3'-end of the BglII site. The length of the rRNA-protected fragment indicated that the 3'-end of 28Sα rRNA is located at 23 nucleotides downstream of the BglII site (Fig. 3B lane 1 and 2). These, combined with the sequence data, demonstrated the gap

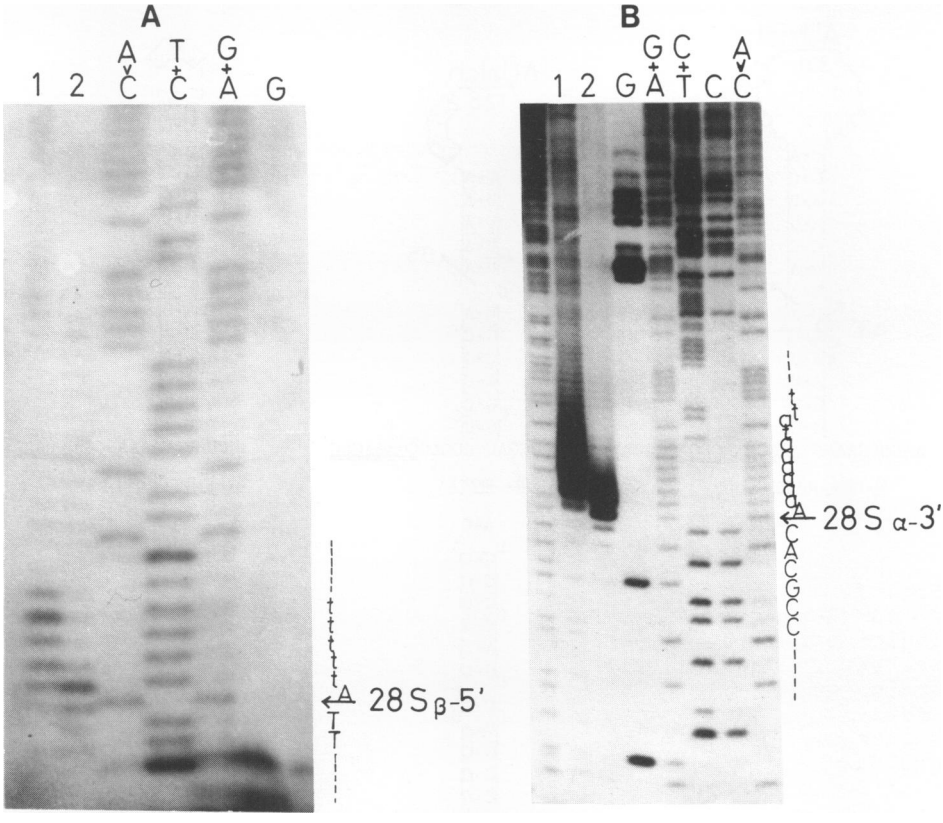

Fig. 3. S1 nuclease mapping of the 5'-end of 28Sβ rRNA (A) and the 3'-end of 28Sα rRNA (B) of B.mori. The 5'- (A) and 3'-labeled (B) single stranded HinfI-BglII 80b fragment (Fig. 1), were hybridized to Bombyx rRNA and treated with S1-nuclease (lane 1 in A and B = 0.1u/µl and lane 2 in A and B = 1u/µl). S1-Nuclease resistant fragments were analysed on 8% polyacrylamide gels containing 7M urea adjacent to sequencing ladders of the original fragments. The arrows indicate the presumed 5'- or 3'-terminal nucleotides.

sequence of 30 nucleotides of the Bombyx hidden break, which is excised from a precursor to the 28S rRNA as a final step of the processing event (Fig. 2).

Discussion

Ware et al. pointed out that the gap region is located within the eukaryotic rRNA-specific expansion segment 5 (7), which is various in both size and base composition among different

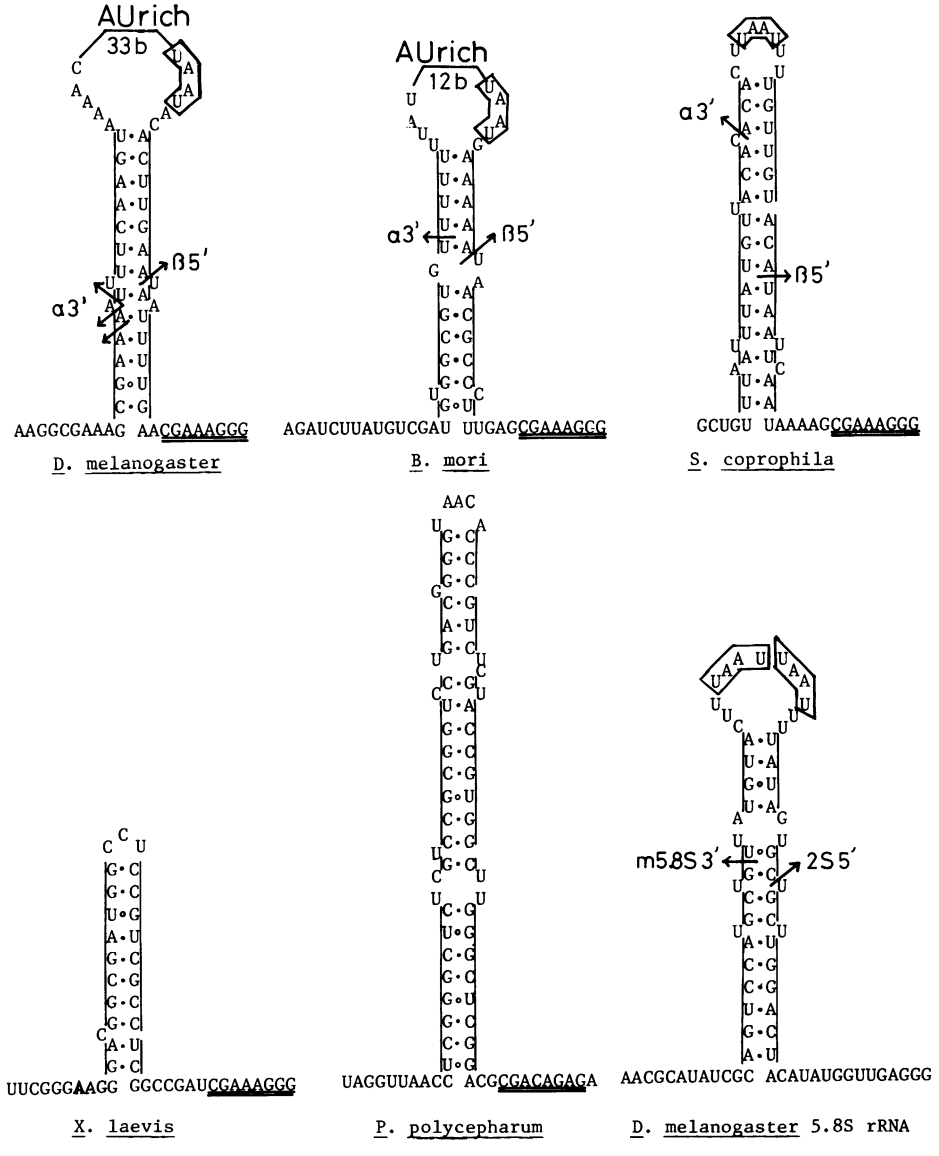

Fig. 4. Comparison of secondary structure models for several expansion segments 5 of 28S rRNA and the processed region of Drosophila 5.8S rRNA. Arrows indicate the processing sites (α3'; the 3'-end of 28Sα, ß5'; the 5'-end of 28Sß, m5.8S3'-; the 3'-end of m5.8S rRNA, 2S5'; the 5'-end of 2S rRNA (17)). The conserved UAAU tract (see text) was boxed in the loop regions of 28S rRNAs and Drosophila 5.8S rRNA. The highly conserved sequence in all the 28S rRNAs was doubly underlined.

species. Since the area flanking the expansion segment 5 is highly conserved throughout eukaryotes and prokaryotes, it is possible to compare the expansion segments themselves whether or not they contain the gap region in them.

In Bombyx rDNA, the expansion segment 5 corresponded approximately to the region between the third BglII and fourth HinfI site (Fig. 2). Comparison of such the expansion segments 5 of different organisms drew structural features of interest as follows: (Ⅰ) All the expansion segments reside precisely at the same region along the 28S molecule. (Ⅱ) The expansion segment 5 of insects contains an extremely AU-rich tract in the gap region while those rRNAs without the hidden break are GC-rich throughout the corresponding segments. (Ⅲ) The sizes and nucleotide sequences of the gap region are very various among insects, although the base composition is equally AU-rich. Thus, there arises one possibility that the AU-rich tract in the expansion segment 5 is essential to the introduction of the hidden break.

Depicted in Fig. 4 are possible secondary structures of expansion segments 5 of the 28S rRNAs from three insects, Physarum (15) and Xenopus when adapted to the Clark's model (16) for Xenopus 28S rRNA so as to maximize intramolecular base-pairings. Also included in the figure is a secondary structure model for Drosophila 5.8S rRNA which was reported previously by Pavlakis et al (17). It has been shown that in dipteran 5.8S rRNA an AU-rich stretch is removed to split the molecule into the two (m5.8S RNA (123b) and 2S RNA (30b)).

It should be emphasized that in both insect 28S rRNAs and Drosophila 5.8S rRNA cleavage seems to occur in the double stranded stem, particularly at an unpaired site intervening paired bases. As a result, a short base-paired (5-8 bp) stem should be left there, which will be involved in the intermolecular interaction between the two subunits thus formed. The processing mechanism presumed above is reminiscent of maturation of E.coli 23S rRNA by means of RNase III (18). In search of signals for this processing, it was found that part of the gap region may invariably form a very AU-rich loop on top of the stem. In contrast with this, the hairpin structure of Xenopus and

Physarum rRNAs which are not subjected to the processing, was GC-rich throughout (Fig. 4).

What is more significant was that the loop, thus formed, always contains the sequence UAAU at about 10b upstream of the 5'-end of 28Sβ. The same sequence was totally missing in any expansion segment 5 of Xenopus, Physarum and yeast rRNA. Further, this 4b tract was also found in the loop of the gap region of Drosophila 5.8S rRNA (but not in Sciara 5.8S rRNA, where UAUU is a relevant sequence (19)). Such a tract was not observed at all in the corresponding region of other 5.8S rRNAs so far studied which do not undergo the cleavage reaction (20).

Not only presence of common structures in the hidden break region of insect 28S rRNA and in dipteran 5.8S rRNA, but also absence of these structures in 28S species without the hidden break and common 5.8S molecules, may be taken to indicate that specific signals essential to the processing events have been generally conserved. However, it remains quite an enigma why, from an evolutionary viewpoint, the hidden break is limited within the 23-28S rRNAs of protostomes (5) and higher plant chloroplasts (4).

Acknowledgements

The work was supported in part by a research grant from the Ministry of Education, Science and Culture of Japan (59106003).

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