Doklady Biological Sciences, Vol. 404, 2005, pp. 348–351. Translated from Doklady Akademii Nauk, Vol. 404, No. 1, 2005, pp. 131–135.

GENERAL BIOLOGY

Characteristics of the Structure of Minor Variants of External Transcribed Spacers of Ribosomal DNA of the Cockroach *Blattella germanica*

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Presented by Academician Yu.P. Altukhov December 30, 2004

Received December 30, 2004

Minor variants of the external transcribed spacer, which have certain structural differences from the repeats characteristic of this species, were found in the genome of the cockroach *Blatella germanica.* The nature of the structural differences corresponds to that characteristic of interspecific variability. Models of evolutionary variability of a cluster of ribosomal genes are discussed in the light of these data.

The cluster of ribosomal DNA (rDNA) representing tandemly repeated genes of ribosomal RNAs (18S, 5.8S, and 28S-like) separated by internal transcribed spacers (ITS1 and ITS2) and external transcribed and nontranscribed spacers (ETS and NTS, respectively) is an integral part of the genome of any eukaryotic organism (except for some protozoans [1]) [2]. The scheme of structural organization of rDNA is shown in Fig. 1.

The rDNA cluster of insects (in particular, cockroaches) contains several hundreds of repeated structural–functional units and, therefore, is a typical example of a multigenic family.

A characteristic feature of multigenic families is the homogeneity of repeated structural units within a species and their dissimilarity in representatives of different species [3]. Today, a common explanation to this characteristic feature of the structural organization of multigenic families is as follows. The concerted nature of evolutionary variability of members of multigenic families is determined by recombination occurring between repeats, with intrachromosomal gene conversion playing the key role in this process [4, 5]. Due to unequal recombination exchange, mutant variants of repeated structural units are eliminated, leading to the uniformity of the members of a multigenic family.

A multigenic family of a new species, differing from that of the ancestral form, originates at the population level. Computer simulation and respective mathematical calculations showed that, if a multigenic family determines a selectively neutral trait, a multigenic family of a new type is formed due to stochastic processes; i.e., one of mutant variants may randomly become the major member of a multigenic family of a newly formed species [3, 4]. The selective significance of the trait does not alter the nature of formation of the new multigenic family but only accelerates this process due to selection pressure. Thus, the suggested models conceptually fit the general theory of neutral evolution [6].

From our standpoint, an alternative mechanism leading to the formation of a multigenic family differing form the ancestral form may be saltational reorganization of this genomic region in reproductive-tract cells. Note that, in this case, an individual rather than a population will represent an evolutionary unit and the basis for formation of a new species.

This approach agrees well with the postulates of the genetic monomorphism theory, according to which the formation of a new species is regarded as the result of large-scale genetic reorganization marked by monomorphic signs rather than a gradual probabilistic process taking place at the population level [7].

Experimental data confirming the possibility of a large-scale rDNA reorganization were obtained in the study of mutant *Drosophila* with large deletions in the cluster of ribosomal genes [8]. It was shown that, at certain combinations of parental phenotypes, hereditable restoration of the number of copies of rDNA repeats is observed in reproductive-tract cells of a part of first-generation progeny. It was also shown that this restoration is determined by the magnification of one or several repeats, which leads to an increase in the number of repeated units and restoration of the multigenic family characteristic of this species [9].

In our opinion, experimental evidence for the existence of a genetic mechanism by which the magnification of some members of a multigenic family is induced, is extremely important for understanding not only the principles of maintenance of the required number of repeats in multigenic families of a certain species

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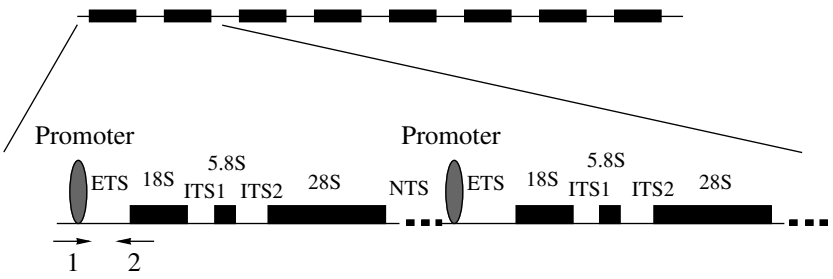

Fig. 1. Scheme of a cluster of eukaryotic ribosomal RNA genes. Designations: NTS, nontranscribed spacer; ETS, external transcribed spacer; ITS1 and ITS2, internal transcribed spacers; 18S, 5.8S, and 28S, respective genes of ribosomal RNAs. Dark oval marks the RNA polymerase I promoter.

but also the trends in formation of multigenic families of a new species.

It can be assumed that the isogenization of repeats proceeds nonuniformly along the multigenic family length and that the flanks of the cluster are involved in this process to a lower extent. In addition, it cannot be rules out that orphons (rDNA fragments located beyond the cluster of ribosomal genes) may serve as genetic material for further evolution [10]. In these regions, new structural variants that may have selective advantage under changed environmental conditions may be formed without a significant effect on the phenotype of an individual.

Selective magnification and targeted gene conversion are two mechanisms that allow a new structural variant to become the major member of a multigenic family or to form a new multigenic family.

It is known that the rate of rDNA synthesis, which is largely determined by the structure of spacer sequences, is correlated with the general level of metabolism, which, in turn, is a key component determining the adaptive potential of an individual for certain environmental conditions. Different levels of metabolism intensity and, as a consequence, different rates of rDNA synthesis may be more adaptive during the occupation of new ecological niches. Today nothing is known on the molecular mechanisms by which magnification is induced. At the same time, taking into account the above speculations, two situations may exist: (1) the number of adaptive rDNA repeats dramatically decreases and (2) the number of repeats corresponds to the average number characteristic of the species, but the type of repeats is not adaptive under the new environmental conditions. For an organism as a whole, magnification of rDNA repeats of a certain type may occur in either case.

Earlier, we performed a comparative analysis of the structure of rDNA ETSs of several closely related cockroach species of the genus *Blatella.* We showed that the structure of ETSs is a species-specific monomorphic trait. When comparing ETSs of different species, we observed characteristic differences consisting predominantly in changed number of subrepeats in ETSs [11].

With regard for the above facts, it can be expected that the genome of cockroaches (especially sibling species) may contain minor ETS variants that, first, exhibit certain structural differences from the repeats characteristic of the species. Second, the type of differences should correspond to that characteristic of interspecific variability. The goal of this study was to detect and analyze minor ETS variants of the cockroach *Blattella germanica*.

ETSs of the cockroach *Blattella germanica* were amplified using the primers that were described earlier—*1* (catcatcttggttagactgtc) and *2* (gtgagactgaaccaagtgtg). Approximate location of the primers in rDNA is shown in Fig. 1. Standard amplification was performed using the kit form Promega according to the protocol provided by the manufacturer. The reaction medium contained 0.1 μg of DNA extracted from whole cockroaches, 1.5 mM $MgCl_2$, and 1 mM of each

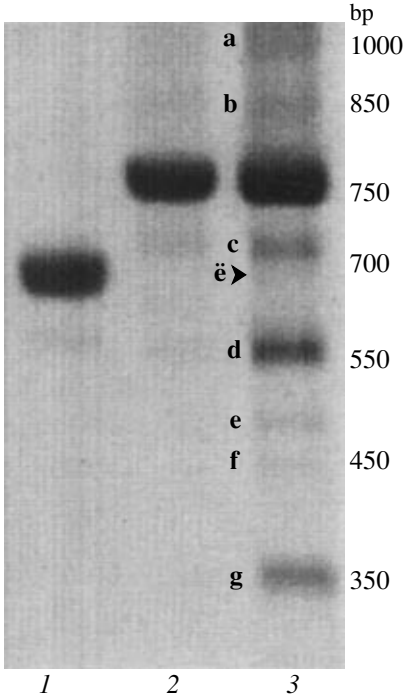

Fig. 2. Amplification of the ribosomal DNA fragment containing an external transcribed spacer (ETS) of (*1*) *B. asahinai* and (*2, 3*) *B. germanica.* PCR enhancers were present in lanes *1* and *2* and absent in lane *3*. letters designate the minor ETS variants.

> *B. asahinai* (the major variant)

GTGAGACTGAACCAAGTGTGTGCCTTCGGGCATGAATGAGTTGGGGCAATGCGTGGAAGGTTCGTCCGAGATATGGTCGA
TCCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACA**CGTACGGTTCTAGCTTGTCTCGG**
CATATCTGTCCGTATAGGATTGTGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCCTT
CGGGCATGAATGAGTTGGGGCAATGCGTGGAAGGTTTGTCCGAGATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGATG
GACGGGGGTAGATCGGGAAAAGAATCCGACA**CGTACGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTG**
TGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCGATCTAGTCTCTCCGCAAGGACCTTTCCC
GTCTCGAGATGTATTTCTTGTGTCCGGGGTCTATAGGTTTTCTTGTCGGCTGTCGGACTTTTTCTGTCGCAGAGTATTAA
TGACTAGGTTGCCATGCGGGGCTTTTGTACCCGTGGCGCCTGTTCGGAGCAcgtgtaaaacaacgcacgagttccctggttGATCCTG
CCAGTAGTCATATG

> *B. germanica* (the major variant)

GTGAGACTGAACCAAGTGTGTGCCTTCGGGCATGAATGAGTTGGGG**CGTACGGTTCTAGCTTGTCTCGGCATATCTGTCC**
GTATAGGATTGTGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCCTTCGGGCATGAAT
GAGTTGGGGCAATGCGTGGAAGGTTCGTCCGAGATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAG
ATCGGGAAAAGAATCCGACA**CGTACGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGTGTGAGACG**
TGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCCTTCGGGCATGAATGAGTTGGGGCAATGCGTGGAAGGTTT
GTCCGAGATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACA**CGTACG**
GTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAG
TGTACTCTGTGCGCGATCTAGTCTCTCCGCAAGGACCTTTCCCGTCTCGAGATGTATTTCTTGTGTCCGGGGTCTATAGG
TTTTCTTGTCGGCTGTCGGACTTTTTCTGTCGCAGAGTATTAATGACTAGGTTGCCATGCGGGGCTTTTGTACCCGTGGC
GCCTGTTCGGAGCAcgtgtaaaacaacgcacgagttccctggttGATCCTGCCAGTAGTCATATG

> *B. germanica* (d)

GTGAGACTGAACCAAGTGTGTGCCTTCGGGCATGAATGAGTTGGGG**CGTACGGTTCTAGCTTGTCTCGGCATATCTGTCC**
GTATAGGATTGTGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCCTTCGGGCATGAAT
GAGTTGGGGCAATGCGTGGAAGGTTCGTCCGAGATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAG
ATCGGGAAAAGAATCCGACA**CGTACGGTTCTAGCTTGTCTCGGCATATCTGTCCGTATAGGATTGTGTGTGTGTGAGACG**
TGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCGATCTAGTCTCTCCGCAAGGACCTTTCCCGTCTCGAGATG
TATTTCTTGTGTCCGGGGTCTATAGGTTTTCTTGTCGGCTGTCGGACTTTTTCTGT CGCAGAGTATTAATGACTAGGTTG
CCATGCGGGGCTTTTGTACCCGTGGCGCCTGTT^{a}GGAGCAcgtgtaaaacaacgcacgagttccctggttGATCCTGCCAGTAGTCATATG

> *B. germanica* (f)

GTGAGACTGAACCAAGTGTGTGCCTTCGGGCATGAATGAGTT^{t}GGGCAATGCGTGGAAGGTT·GTCCG^{c}GATATGGTCGAT
CCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCG^{a}GAAAAGAATCCGACA**CGTACGGTTCTAGCTTGTCTCG·CA**
TATCTGTCCGTA··GGATTGTGTGTGTG··AGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCG^{t}GATCTAG
TCTCTCCGCAAGGACCTTTCCCGTCTCGAGATGTATTTCTTGT··CCGGGGT^{g}TATAGGTTTTCTTGTCGGCTGTCGGACT
TTTTCTGTCGCAGA^{c}TA^{c}TTAATGACTAGGTTGCCATGCGGG^{a}CTTTTG^{c}ACCCGTGGCGCCTGTTCGGAGCAcgtgtaa^{c}aca
^{g}cgcacgagttccctgg·ttGATCCTGCCAGTAGTCATATG

> *B. germanica* (g)

GTGAGACTGAACCAAGTGTGTGCCTTCGGGCATGAATGAGTTGGGG**CGTACGGTTCTAGCTTGTCTCGGCATATCTGTCC**
GTATAGGATTGTGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCGATCTAGTCTCTCC
GCAAGGACCTTTCCCGTCTCGAGATGTATTTCTTGTGTCCGGGGTCTATAGGTTTTCTTGTCGGCTGTCGGACTTTTTCT
GTCGCAGAGTATTAATGACTAGGTTGCCATGCGGGGCTTTTGTACCCGTGGCGCCTGTT CGGAGCAcgtgtaaaacaacgcacg
agttccctggttGATCCTGCCAGTAGTCATATG

> *B. germanica* (ë)

GTGAGACTGAACCAAGTGTGTGCCTTCGGGCATGAATGAGTT^{t}GGGCAATGCGTGGAAGGTTCGTCCGAGATATGGTCGAT
CCCGCTTGAGGCTGCAGAGCCGATGGACGGGGGTAGATCGGGAAAAGAATCCGACA**CGTACGGTTCTAGCTTGTCTCGGC**
ATATCTGTCCGTATAGGATTGTGTGTGTGTGAGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCCTTC
GGGCATGAATGA^{a}GTTGGGGCAATGCGTGGAAGGTTTGTCCGAGATATGGTCGATCCCGCTTGAGGCTGCAGAGCCGATGG
ACGGGGGTAGATCGGGAAAAGAATCCGACA**CGTACGGTTCTAGCTTGTCTCG·CATATCTGTCCGTA··GGATTGTGTGT^{c}T**
G··AGACGTGGCCTGTGACCGACCCCACAAAGAGTGTACTCTGTGCGCGATCTAGTCTCTCCGCAAGGACCTTTCCCGTCT
CGAGATGTATTTCTTGTGTCCGGGGTCTA··GGTTTTCTTGTCGGCTGTCGGACTTTTTCTGTCGCAGAGTATTAATGACT
AGGTTGCCATGCGGGGCTTTTG^{c}ACCCGTGGCGCCTGTTCGGAGCAcgtgtaa^{c}acaacgcacgagttccctggttGATCCTGCCAGTAG
TCATATG

Fig. 3. Comparison of the nucleotide sequences of different variants of the external transcribed spacer of closely related cockroach species. Double underlining indicates the primers used for the amplification of the region studied. Dark background, bold font, and single underlining mark three types of subrepeats. Small font marks the beginning of the 18S gene. Superscript letters designate the nucleotide substitutions; spaces show deleted nucleotides. Letters d, f, g, and ë designate the fragments corresponding to those in Fig. 2.

of dNTP. Amplification included (1) denaturation (95°C, 5 min); (2) 30 cycles of denaturation (94°C, 1 min), annealing of primers (55°C, 2 min), and elongation (72°C, 3 min); and (3) final elongation (7 min). The result of amplification of ETSs of *B. asahinai* and *B. germanica*, which was performed under the standard conditions described above, is shown in Fig. 2 (lanes *1* and *2*, respectively). Electrophoresis in 1% agarose gel showed that the amplified rDNA preparation contained only one fraction, whose size, as shown earlier [11], is strictly species-specific.

To amplify the minor ETS fractions of cockroach rDNA, the reaction mixture was supplemented with PCR enhancers (sodium betaine and ammonium sulfate; final concentrations, 1 M and 15 mM, respectively), and the final concentration of $MgCl_2$ was increased to 5 mM. In other cases, the GenePakCore kit (Promega) as used, which contains PCR enhancers. As a result of amplification under these conditions, additional DNA fragments were detected (Fig. 2, lane *3*, designated with letters). The additional fragments *d, f*, and *g* were cloned into the plasmid pGEM-T Easy Vector (Promega), and their nucleotide sequence was determined. In addition, during cloning the specified DNA fragments, an additional fragment *e* coinciding in size with the major ETS variant of *B. asahinai* was cloned.

Figure 3 shows the nucleotide sequence of the major ETS variants of the two sibling species and the minor ETS of *B. germanica.* A comparison of the nucleotide sequence of the major and minor variants of *B. germanica* showed that the minor variants derived from the major variant characteristic of this species by deletion of extended DNA regions consisting of a series of subrepeats. Note that the structural differences between the major and minor ETS variants of the same species are similar to the differences observed between the major ETS variants of closely related cockroach species of the genus *Blatella* (*B. germanica* and *B. asahinai*). In all cases, the differences in the ETS structure consisted in a deletion of some subrepeats; the minor fragment *e* is nearly identical to the major variant of *B. asahinai* ETS.

It is known that amplification of subrepeat-containing DNA fragments may be accompanied by the formation of recombinant PCR products *in vitro* as a result of annealing of a more extended, partly elongated single-stranded sequence instead of a primer during one of the first amplification cycles [12, 13]. Taking into account this fact, the detection of the minor ETS variants in this study could be due to insufficient inaccuracy of PCR. However, the differences in the structure of the major ETS variants of two sibling species cannot be interpreted as the result of artifact amplification. Additionally, from our standpoint, the detection of the fragment *f* containing multiple point substitutions (Fig. 3) is a principal moment. The amplification of this fragment cannot be explained by the formation of a recombinant sequence *in vitro*, because each subrepeat of this ETS variant and the adjacent region contain multiple nucleotide substitutions. In our opinion, the detection of the minor fragment *e*, identical to the major ETS variant of *B. asahinai* with respect to the structure of subrepeats (except for several nucleotide substitutions), is also very important.

The experimental data obtained in recent years convincingly testify to the functional importance of spacer rDNA sequences [14]. It can be assumed that the number of subrepeats within ETSs affects the rate of rDNA synthesis from the promoter located adjacent to this spacer sequence (Fig. 1). This assumption will be tested experimentally in our further studies.

It is known that the evolution of cockroaches of the genus *Blatella* was accompanied by the occupation of new ecological niches for which different levels of metabolism intensity are adaptive. The minor ETS variants described may represent material for further evolution of the species and form new variants of rDNA clusters.

ACKNOWLEDGMENTS

This study was supported by the Program of Basic Research of the Presidium of the Russian Academy of Sciences "Plant, Animal, and Human Gene Pool Dynamics" (project no. 24P-IOG-09-2004).

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