Histone and ribosomal RNA repetitive gene clusters of the boll weevil are linked in a tandem array

R. Roehrdanz*, L. Heilmann‡, P. Senechal‡§, S. Sears* and P. Evenson*¶

*Biosciences Research Laboratory, Red River Valley Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, and †North Dakota State University, Dept of Biochemistry, Fargo, ND, USA

Abstract

Histones are the major protein component of chromatin structure. The histone family is made up of a quintet of proteins, four core histones (H2A, H2B, H3 & H4) and the linker histones (H1). Spacers are found between the coding regions. Among insects this quintet of genes is usually clustered and the clusters are tandemly repeated. Ribosomal DNA contains a cluster of the rRNA sequences 18S, 5.8S and 28S. The rRNA genes are separated by the spacers ITS1, ITS2 and IGS. This cluster is also tandemly repeated. We found that the ribosomal RNA repeat unit of at least two species of Anthonomine weevils, Anthonomus grandis and Anthonomus texanus (Coleoptera: Curculionidae), is interspersed with a block containing the histone gene quintet. The histone genes are situated between the rRNA 18S and 28S genes in what is known as the intergenic spacer region (IGS). The complete reiterated Anthonomus grandis histone-ribosomal sequence is 16 248 bp.

Keywords: histones, ribosomal RNA, Anthonomus, repetitive sequences, internal transcribed spacers.

Introduction

The histones and ribosomal RNAs (rRNA) comprise two families of genes that are critical to eukaryotic existence.

Histone genes code for a set of basic proteins that are the major constituents of chromatin. They are involved in both DNA packaging into nucleosomes and regulating gene expression. The histone family can be divided into two groups, four core histones (H2A, H2B, H3, H4) and linker histones (H1). Because of the cells’ constant requirement for histones, the genes are reiterated with a hundred or more copies of each gene. Among invertebrates the histone genes are typically clustered as quartets (core histones) or quintets (core histones plus H1). The quintet arrangement is most prevalent in the insects that have been examined. The clusters are usually linked to create tandemly repeated regions of the genome (Schiemann et al., 1998; Nagel & Grossbach, 2000; Nagoda et al., 2005).

The rRNA genes share the tandemly repeated organization structure with the histones. In all eukaryotes three rRNA genes, 18S, 5.8S and 28S, are linked as a common transcriptional unit separated by a non-transcribed spacer in the tandem array (Tautz et al., 1988; Hankeln & Schmidt, 2001). Like the histones there can be 100 or more copies per cell. A fourth rRNA, the 5S rRNA, is also repeated but is usually not linked to the other three (Drouin & de Sa, 1995).

We report the first examples, in two Anthonomine weevils, of the fusion of these two vital gene families. The histone and rRNA gene cluster have become integrated so that a histone quintet alternates with an rRNA triplet in an extended tandem repeat where each unit is more than 16 kb in length. The position and polarity of the histone genes in the quintet is also a new arrangement for insects. The details of this unusual organization are described for the cotton boll weevil, Anthonomus grandis (Coleoptera: Curculionidae). We also show that a closely related weevil contains the same tandem repeat.

Results and discussion

The complete tandem sequence encompassing the boll weevil histone and rRNA gene blocks is 16248 bp. The distribution and orientation of the genes is shown in Fig. 1. The size of each gene or region is given in Table 1. ITS1 and ITS2 are the spacers between the 18S rRNA and 5.8S rRNA, and the 5.8S rRNA and 28S rRNA, respectively.
ITS1 and ITS2 are found in all rRNA repeats and have been widely used in phylogenetic studies. The spacer region between the 28S and 18S rRNAs has been designated the intergenic spacer region or IGS (Tautz et al., 1988). We have used that terminology and have separated the IGS into two components, IGS1 and IGS2, as shown in Fig. 1 and Table 1. Each part of the IGS region flanks both rRNA genes and histone genes. The orientation of the histone genes has H2B, H4 and H1 in the forward direction with H2A and H3 in the reverse direction. In addition, there are four spacer regions located between the histone genes. The histone spacers have had some limited use in phylogenetics (Yang et al., 2004). The span of the rRNA genes is 7176 bp which compares favourably with the 7233 bp for these genes in *Drosophila melanogaster* (M21017). The span of the histone genes at 4316 bp is within the range reported for other insects, 4033 bp in *Drosophila americana* (AB192418) to 5192 in *Drosophila orena* (AB105181).

There appear to be at least three genetically distinct populations of weevils, the south-eastern boll weevil, the thurberia boll weevil and the Mexican boll weevil (Burke et al., 1986). At the molecular level these populations can be distinguished by mtDNA and ITS2 differences (Roehrdanz, 2001; Senechal, 2004). The DNA for the sequence originated with boll weevils derived from the south-eastern population. To establish that this genome organization was not an artifact restricted to weevils that had been reared in a colony for many years, PCR amplification was performed using template from wild caught weevils and primers located within the identifiable genes. Weevils from all three populations were included. Their population status had been previously defined by sequencing the ITS2 region (primers AGHR5.8SF and AGHR28S) (Senechal, 2004). Primers AGHR37-AGHR82 are in the 18S and 28S rRNAs and amplify the entire intergenic region (~9400 bp). Primer AGHR82 in the 28S rRNA combined with primer AGHR13 in histone H2A amplifies the IGS1 region (~2700 bp). Primers AGHR50 and AGHR20 amplify a segment entirely within the histone genes from H3 to H1 (~2500 bp) (Fig. 2).

The expected products were obtained from all three *A. grandis* populations. Collectively the amplicons connect the conjoined rRNA and histone genes on both ends and confirm both the gene order and the tandem array of the gene clusters (Fig. 3).

If the conjoined rRNA and histone genes are nonfunctioning pseudogenes whose inactivity is a consequence of the original splicing event, it would be expected that random substitution would occur over evolutionary time resulting in sequences that have diverged substantially from the functional genes of other insect species. The 18S and 28S rRNAs were compared directly with sequences...
from the database and Minimum Evolution trees were constructed (Figs 4 and 5). The entire 18S sequence was used. Because the 28S gene contains regions that are somewhat conserved interspersed with highly divergent segments, a segment of 1000 bp from a conserved region near the 5’ end was used for comparisons. In both cases the boll weevil (BW) sequences cluster with other weevils (Curculionidae). The Curculionidae clades have good bootstrap support, 96% for 18S and 100% for 28S. The base substitution rate within the weevils (Curculionidae) for the 18S gene is <0.01. The boll weevil 18S is most similar to the 18S sequence from Anthonomus peticularis, with a bootstrap support for that pairing of 99%. For the 28S region examined the substitution rate within the weevils is much higher, 0.05, but the rate between the weevils and other Coleoptera is also higher, >0.08. This evidence supports the premise that the boll weevil rRNA sequences are legitimate and from functional genes.
expected to show more divergence and be easily recognized. In *Chironomus* an orphan H1 gene is the result of a translocation and has diverged considerably from the normal H1 gene sequence (Hankeln & Schmidt, 1993). At the amino acid level the BW H3 and H4 translation products are identical to those from Diptera, H2B differs by only two amino acids from *Drosophila*, and H2A by only four amino acids from *Drosophila*. Histone H1 is the most variable of the five genes. There were no H1 sequences from other Curculionidae in the database, but the BW H1 was most similar to the sequence from another Coleoptera as opposed to several Diptera species (data not shown). Together the nucleotide and amino acid sequence data indicate that the tandem histone-rRNA genes are the functional genes.

Among the insects the typical arrangement of the histone genes is a repeating quintet comprising the four core histones (H2A, H2B, H3, and H4) along with histone H1. Partial exceptions to this rule have been noted in *D. americana* and *D. virilis*. In both of those species a repeating quartet consisting of only the core histones coexists with the quintet repeat (Schienman et al., 1998; Nagel & Grossbach, 2000; Nagoda et al., 2005). Nagoda et al. (2005) hypothesize that the quartet is derived from the quintet via deletion of H1. Since the quartet arrangement is viable in many other invertebrates (Baldo et al., 1999; Barzotti et al., 2000; Eirín-López et al., 2004) it may be more common in insects than has been recognized. The PCR primers used here demonstrate the link between H1 and the core histones but cannot rule out the presence of quartets as well.

In the insect histone gene cluster, H2A and H2B are adjacent and transcribed in opposite directions. Genes H3 and H4 constitute a similar oppositely transcribed pair.
In the mussel *Mytilis* genes H3 and H4 are both transcribed in the same direction and in some cases have H1 interposed between them (Albig et al., 2003; Eirín-López et al., 2004). The relative polarity of the two linked pairs to each other and to H1 is variable. Figure 6 shows several insect arrangements. For ease of comparison the clusters are all portrayed with histone H1 oriented to the right and at the right end. In *Anthonomus* and *Rhyncosciara/Chironomus* H1, H2B and H4 are transcribed from the same strand (Hankeln & Schmidt, 2001). This contrasts with the *Drosophila* clusters where H1, H2A and H3 are transcribed from the same strand (Kremer & Hennig, 1990; Schienman et al., 1998; Matsuo, 2000; Nagel & Grossbach, 2000; Tsonemoto & Matsuo, 2001; Kakita et al., 2003; Nagoda et al., 2005). The *Apis* histone cluster reflects a fourth arrangement of the quintet. H1, H2B and H4 have the same polarity as *Anthonomus*, but the H3/H4 and H2A/H2B pairs have switched positions relative to H1. Of the eight possible arrangements of H1 and the core pairs, four have been identified in an extremely small taxonomic sampling where 11 of 15 available insect histone gene arrays are from *Drosophila* and two more are other Diptera (Fig. 6). Those arrangements are *Drosophila* (H2BH2A)(H4H3)(H1), BW (H2AH2B)(H3H4)(H1>, *Apis* (H4H3)(H2AH2B)(H1), and *Chironomus* (H3H4)(H2AH2B)(H1). It seems likely that the other four arrangements (H2BH2A)(H3H4)(H1>, (H2AH2B)(H4H3)(H1>, (H4H3)(H2BH2A) (H1>, and (H3H4)(H2BH2A)(H1>) could occur in the huge class of insects.

The IGS1 and IGS2 regions were scanned for possible repetitive elements. Both spacers contain numerous small direct and inverse repeats of 8 bp or less that had no apparent pattern to their distribution. The 5’ half of IGS1 contains an elaborate complex of direct repetitive units (DR), some of which overlap each other (Fig. 7). The largest of the repeats, DR-1, is 270 bp and the two copies overlap by about 50%. Within the span of these two repeats is a set of three 145 bp overlapping repeats, DR-1A. DR-1C has four identical 61 bp copies associated with the two larger repeats along with two somewhat degraded copies 5’ to the complex. The first sequence (most 5’) differs from the four identical sequences by 9 bp while the second differs by 4 bp. Quartets of other smaller repetitive sequences can be identified in the main complex and some have additional copies either upstream or downstream. DR-5 is a set of three evenly spaced 16 bp repeats situated 5’ to the main complex. The middle one differs from the other two by a single substitution. The region also has the potential for some larger stem-loop configurations (SL). The smaller one, SL1, is 60 bases that can potentially form a hairpin with a terminal 7 nucleotide loop. SL2 and SL3 could fold into a stem-loop with an 18 bp stem connecting to a 107–108 base loop. With no data to indicate whether these repeat motifs and possible secondary structures are conserved in other his-ribo linkages, it is premature to ascribe any regulatory function to them.

The histone-rRNA repeat cluster is found in three identifiable populations of *A. grandis* but is it unique to that species, is it found in other *Anthonomus*, or does it have an even broader phylogenetic distribution? PCR was performed on DNA from other species of weevils, *Anthonomus texanus, Anthonomus griseisquamis, Anthonomus somniculosus, Smicronyx sordidus* (grey sunflower seed weevil), *Smicronyx fulvus* (red sunflower seed weevil).
Apion longirostre (hollyhock weevil), and Haplorhynchites aeneus (sunflower headclipping weevil). Primer pairs employed were AGHR20-AGHR50 between histone genes and AGHR37-AGHR23, AGHR13-AGHR92, and AGHR37-AGHR50 to establish linkage between histone and rRNA (Fig. 1). Controls for PCR were mtDNA primers and the ITS1-ITS2 region (AGHR18S-AGHR28S) or ITS2 alone (AGHR5.8SF-AGHR28S).

The DNA from A. texanus yielded the most positive results with all primer pairs except AGHR13-AGHR92 producing amplicons similar in size to those from A. grandis. To verify that the expected histone and rRNA genes were amplified, the ends of the products AGHR20-AGHR50 (H3-H1), AGHR23-AGHR37 (H1-18S) and AGHR5.8SF-AGHR28S (ITS2) were sequenced. The sequences all aligned to the expected positions of the A. grandis long sequence (Fig. 8). The sequence identity of A. texanus regions, corresponding to portions of H3, H4 and H1, ranges from 90.2% to 95.2%. The sequence identity of the H3-H4 spacer is 81.7% which is the same as for ITS2. The IGS2 is more divergent with only 50% similarity in a large section and is not useful for trying to determine if portions of IGS2 derive from the histone cluster, the rRNA gene cluster, or neither. The success of the amplification and the sequence data indicate that A. texanus also carries the histone-rRNA repeat and the arrangement of the rRNA genes relative to H1, H3 and H4 matches that of A. grandis. The H2A-H2B pair is also presumably in the same place but its orientation has not been confirmed. The failure of the primer pair AGHR13-AGHR92 (H2A-28S) could be attributed to either the reverse orientation of the H2A-H2B genes or degradation of the primer binding site in 28S between the two species. The 28S gene can contain either expansion segments or R1 retrotransposons that alter the nucleotide sequence between species and could easily eliminate a primer binding site (Hancock & Dover, 1988; Gentile et al., 2001). Two other Anthonomus weevils, A. griseisquamis and A. somniculus, gave inconclusive results. The AGHR50-AGHR37 primers produced products about 10% larger than in A. grandis and the other three primer pairs did not amplify. The ends of these amplicons were not sequenced.

PCR analysis did not establish the presence of the histone-rRNA linkage in the other weevil species, although some of the results were ambiguous. None of the primers were successful in A. longirostre making it the strongest case for not having the tandem array. Three samples of S. sordidus yielded H3-H1 amplicons about 1000 bp smaller than Anthonomus, a size that could be accommodated by a smaller spacer between H4 and H1. However the three individuals produced different size products for the H3-18S region. One was the size of Anthonomus, one about half that size, and one failed to amplify. Its sister species, S. fulvus, presented similar difficulties. Three individuals had no amplification except for the control genes. Three other samples produced H3-18S products about 40% of the size as Anthonomus and had a weak multi band amplification profile for the other three regions. Haplorhynchites aeneus gave weak ambiguous amplification for two of the primer sets (not shown). Together the results for weevils other than A. grandis and A. texanus cannot be considered definitive because of the potential for rearrangement of the gene orientations and the loss of primer binding sites especially in the rRNA genes.

If the histone-rRNA tandem repeat is restricted to a fraction of the genus Anthonomus, it could be a very useful phylogenetic marker as a shared derived character for taxonomic revision of the genus. Anthonomus is rather expansive containing perhaps 500 species worldwide (Bisby et al., 2009) with some species groups being assigned to subgenera (Burke, 1964; Clark & Burke, 1986). The odds that such a large group of species is monophyletic at the molecular level are probably not very great. An unusual event such as insertion of the histone gene block into the rRNA gene block (or vice versa) could define a lineage. At present, the existence of histone-rRNA arrays in which the histone genes have different orientations cannot be excluded.

Evolutionary evidence indicates that both the rRNA and histone gene clusters can be translocated within the genome. The rRNA genes can be recognized cytologically as the nucleolus organizer region (NOR). The number and chromosomal location of the NORs can vary. In Drosophila the rRNA genes can be found on both the X and Y chromosomes, only the X chromosome, the 4th chromosome, and the Y and 4th chromosomes depending on the species (Roy et al., 2005). Tiger beetle species with multiple sex chromosomes can have NORs on one of three X

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three sets of autosomes (Galián et al., 1995). In the Australian ant genus *Myrmecia*, the 28S rRNA was found on a low of 2 chromosomes to a high of 19 chromosomes depending on the species (Hirai et al., 1996). An examination of 30 Lepidoptera species revealed NOR numbers ranging from 1–11. Single NORs tended to be located medially on the chromosomes whereas multiple NORs were mostly terminally located clusters. The number of rDNA clusters varied even as the number of chromosomes remained constant (Nguyen et al., 2010). Similarly, a survey of the distribution of rDNA loci in 49 grasshopper species found that while most species had 1–3 rDNA clusters, there were six species that contained 5–10 clusters (Cabrero & Camacho, 2008). Together these results indicate that the NORs can move independently of major chromosome fissions or fusions. Histone genes are more difficult to track. In many *Drosophila* the histone quintet is restricted to a single cytological locus, although there are what are described as variant H2A genes at a second site (Fitch et al., 1990; Spradling et al., 1999). Ranz et al. (2003) compared 297 molecular markers between *Drosophila repleta* and *Drosophila buzzatii* and found the frequency of transposition to be very low, only one marker out of 297, but that marker was a histone. In *Chironomus* the histone genes are found at five linked loci on one chromosome and at an isolated location on another chromosome (Hankeln & Schmidt, 1993). So it is clear that both blocks of genes can be transposed around the genome. A single transposition of either the histone or rRNA gene cluster would be sufficient to account for the results.

There are other examples of the translocation and merging of repetitive genes. In lower eukaryotes another ribosomal RNA, the 5S rRNA, has demonstrated a proclivity to become linked with other reiterated genes, the 18S-5.8S-28S rRNA cluster, the trans-spliced leader, and the histone gene cluster. While the majority of these events have been described in fungi, protists and nematodes, the 18S-5.8S-28S rRNA plus 5S rRNA and the histones plus 5S rRNA have been found in crustaceans (Drouin & de Sa, 1995; Barzotti et al., 2000). There appears to be neither obvious advantage nor disadvantage to the association of SS rRNA sometimes with other tandemly repeated families. Indeed Drouin & de Sa (1995) speculate that the 5S genes freely transpose to other genomic locations and the reason they seem to show up so frequently in these other families is the accidental result of these families being of interest to molecular evolutionists and molecular biologists.

The merging of the histone quintet with the rRNA genes in *Anthonomus* may be one of the largest sets of repeated genes to have been unionized in this manner. There is insufficient data and taxa to determine if the linking of the highly conserved histones with the somewhat more variable rRNA genes has exerted a concerted evolutionary effect to dampen the lability of the rRNAs. The phylogenetic utility of this gene rearrangement remains to be determined. A number of questions need answers before any broader significance can be attached to the originating event. It would be informative to know if the transposition is unique to a small set of *Anthonomus* or if it has a more ancient evolutionary history. Likewise, we would want to know if the event has occurred multiple times in the evolution of insects and, if so, are there lineages where the two gene blocks have become separate again.

### Experimental procedures

*Anthonomus grandis* DNA for the primary sequence determination was obtained from bulk specimens of the dark body ebony mutant strain that was being maintained as a laboratory colony in Fargo, ND in the 1980s and 1990s. Additional DNA samples for PCR were obtained from the south-eastern boll weevil, Mexican boll weevil, and thurberia boll weevil collections as described in Roehrdanz (2001). *A. texanus*, *A. grisaeiquamis*, and *A. somniculus* from south central Mexico were provided by R. Jones (UNAM-Queretero, Mexico). Other weevil species were collected around Fargo, ND. A genomic library was constructed from the ebony DNA in λEMBL3 and the library was screened with clones containing either *Drosophila* histone or ribosomal RNA sequences. All but one of the positive boll weevil clones were relatively small and hybridized only to one probe, either the histone genes or the rRNA genes. These clones were not examined further. However, one of the boll weevil clones hybridized to both the histone and rRNA genes. This large fragment was subcloned as a collection overlapping smaller pieces suitable for sequencing. Sequencing was performed from the vector universal sites and by primer walking with newly designed primers. The entire process has been described in detail for a different boll weevil gene (Trewitt et al., 1992).

DNA extraction of individual weevils was carried out using a high salt method (Cheung et al., 1993). Total genomic DNA served as the template for the long PCR and standard PCR amplifications. The details of the long PCR amplification reaction are described elsewhere (Roehrdanz, 1995; Roehrdanz & Degrugillier, 1998). For standard PCR reaction, components were from the Applied Biosciences Taq Gold kit and the PCR conditions were 35 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 3 min. The PCR primers are listed in Table 2, and their placement in the extended sequence is shown in Fig. 1. Some of the PCR amplimers were sequenced in both directions; others were sequenced only from the ends. Where applicable, sequence information from these amplified segments was used to resolve ambiguities in the long sequence. GenBank accession numbers are EU215423 for the complete sequence and AY882992-AY883003 for individual ITS2 regions of *A. grandis*. Except for *A. texanus*, PCR reactions from other weevil species were not sequenced but only examined for the presence and size of product.

Sequence alignments and Minimum Evolution trees were constructed with MEGA4 software (Tamura et al., 2007). Histone and rRNA sequences and gene arrangements from other insects were obtained from GenBank. Diptera: AB105180 (*Drosophila teis-
Primers used to amplify portions of the boll weevil histone-ribosomal RNA repeat

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<th>Primer</th>
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