

Epigenetics Evolution and Replacement Histones: Evolutionary Changes at *Drosophila* H3.3A and H3.3B

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Abstract

Histone modification and replacement in variant histones result in nucleosome remodeling and play an important role in epigenetics. The evolutionary mechanism of epigenetics was investigated by analysing the variant histone 3 genes, H3.3A and H3.3B, in 12 *Drosophila* species. A U11-48K-like gene coding for a zinc finger domain was located in the region upstream of the H3.3A gene in all *Drosophila* species studied. The U11-48K-like and H3.3A genes may be co-regulated, as is the case for the H3-H4 gene pair. However, no strongly conserved nucleotide sequence was found in the 5' - regions of the two genes. Various combinations of gene pairs were found upstream or downstream of the H3.3B gene but none were conserved amongst a broad range of *Drosophila* species. Moderately conserved DNA sequences were found in the H3.3B gene regions around the first intron and outside the coding region of the fifth exon, but DNA sequences in the upstream or downstream regions of the H3.3B genes were not strongly conserved. Different codon usages for three H3 genes (H3, H3.3A and H3.3B) were found for 13 of 18 amino acids and codon usages characteristic of replacement histones (H4r, H3.3A and H3.3B) were found for several amino acids. Several histone modification sites in H3, H3.3A and H3.3B showed extraordinarily biased codon usages. These results suggested the importance of the translational step for histone modification and for the development of epigenetic systems.

Keywords: Epigenetics; Variant histones; H3.3A; H3.3B; *Drosophila*

Introduction

Understanding the evolutionary mechanisms by which phenotypes develop requires studying the systems responsible for the phenotypes. It is now clear that epigenetics play an important role in phenotypic expression and many biological phenomena [1-9]. Both the modification of histones and their replacement with variants causes chromatin remodeling and these are recognized as epigenetic mechanisms [10-11]. Therefore, the evolutionary mechanism underlying epigenetics can be studied by investigating evolutionary changes in the system for histone replacement and modification [12].

Histone 3 is a small (about 135 amino acid) protein and binds histone 4 (H4) to form a H3-H4 dimer [13]. Together with histone 2A (H2A) and histone 2B (H2B), four histones are assembled into the histone core of the nucleosome [13]. In *Drosophila* there are three H3 proteins, a replication-dependent H3 and two variant H3s, H3.3A and H3.3B [14-15]. In contrast to the replication-dependent H3 gene [16-18], the H3 variant genes are single copy genes with introns and are located at *IIL25C3* for H3.3A and at *X8C14* for H3.3B in *Drosophila melanogaster* [15]. Four amino acid substitutions between H3 and the H3 variants have been identified [1,5,19-20]. H3 variants are highly conserved and no amino acid changes have been observed between H3 variants [15]. It is now known that biological 'meaning' is triggered by replacing histones with other histones that may carry different chemical modifications [1-2,21]. Several important questions remain to be answered, including 1) when and how histone variants are replaced, 2) which histones are modified, 3) which amino acids are modified, 4) at which sites amino acids are modified, and 5) what kinds of modifications occur. For example, a single site methylation at K9 (Lys at position 9) of H3 results in 'gene silencing', whereas multiple modifications such as methylation at K4 and acetylation at K9 of H3 result in 'gene expression' [2]. Clearly, the mechanisms of histone modification and replacement are very important [1-2, 21].

It was recently reported that some histone modifications, such as the methylation of H3 Lys9, occur during translation but not after translation [22]. This finding suggested a possible relationship between translation and histone modification. We previously analysed the

structure, genetic variation, and evolution of codon bias and guanine-cytosine content of *Drosophila* histone genes [17-18,23-30]. The evolutionary change of H4r, a replacement for H4, has been reported in *Drosophila* [12]. Here, the variant H3.3A and H3.3B genes from 12 *Drosophila* species were examined to study the mode of evolution of these replacement histones and their effect on the evolution of epigenetics.

Materials and Methods

Data analysis

The nucleotide sequences of the regions flanking and including the histone 3 genes (*H3*, *H3.3A* and *H3.3B*) from each of 12 *Drosophila* species (*D. simulans*, *D. sechellia*, *D. melanogaster*, *D. yakuba*, *D. erecta*, *D. ananassae*, *D. pseudoobscura*, *D. persimilis*, *D. willistoni*, *D. mojavensis*, *D. virilis* and *D. grimshawi*) were obtained from either FlyBase (FB2016 02) or DDBJ (Table 1). Clustal W (ver. 2.1) was used for multiple sequence alignment [31].

Results

Analysis of the genomic organization of the H3.3A and H3.3B genes in twelve *Drosophila* species

In contrast to the tandem cluster array for H3 genes [16-18,23-26,32], H3.3A and H3.3B are single copy genes with introns and are located at *IIL25C3* and *X8C14*, respectively, in *D. melanogaster* (Figure 1) [15]. Genomic arrangements of the H3.3A and H3.3B genes from

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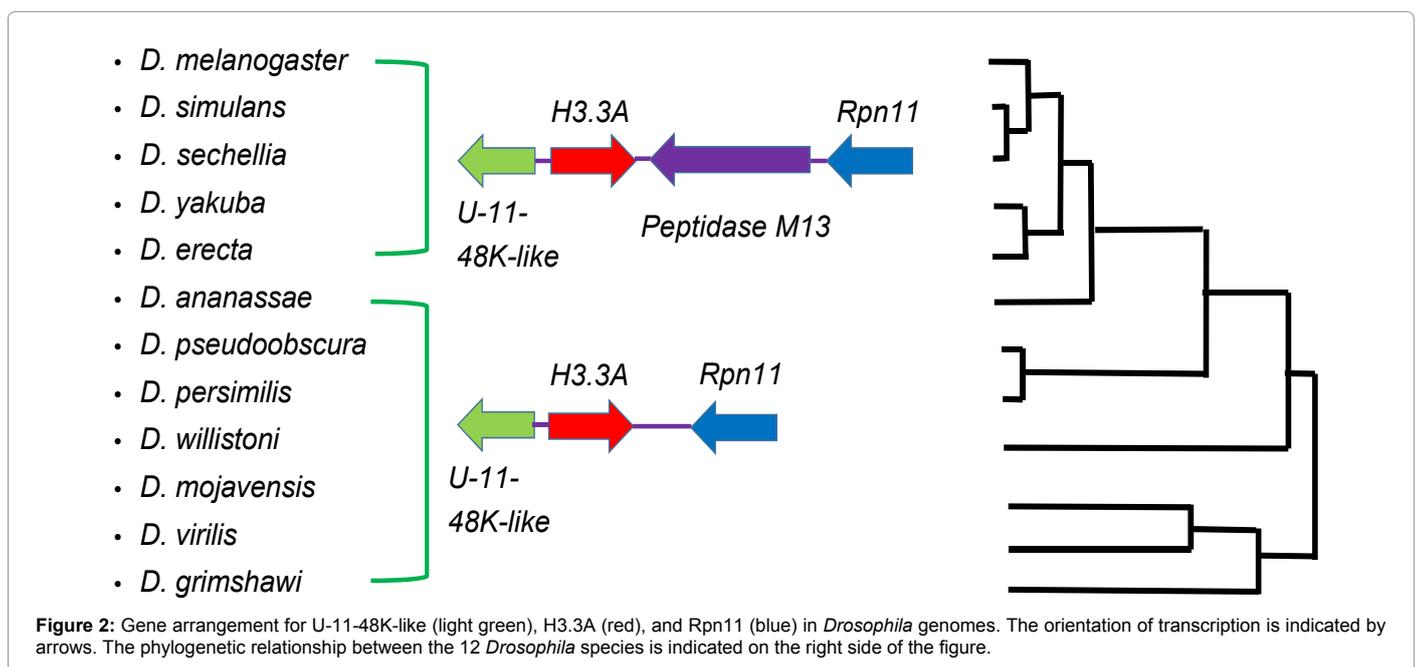
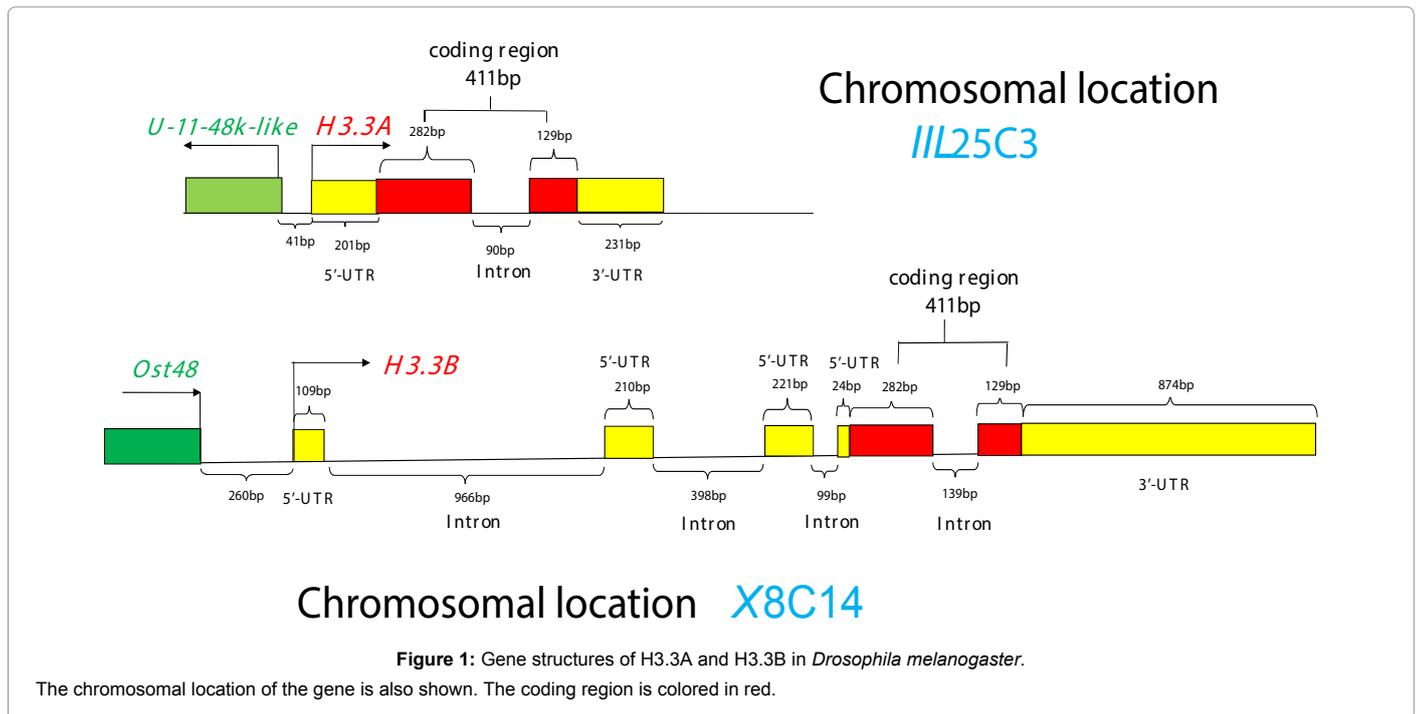
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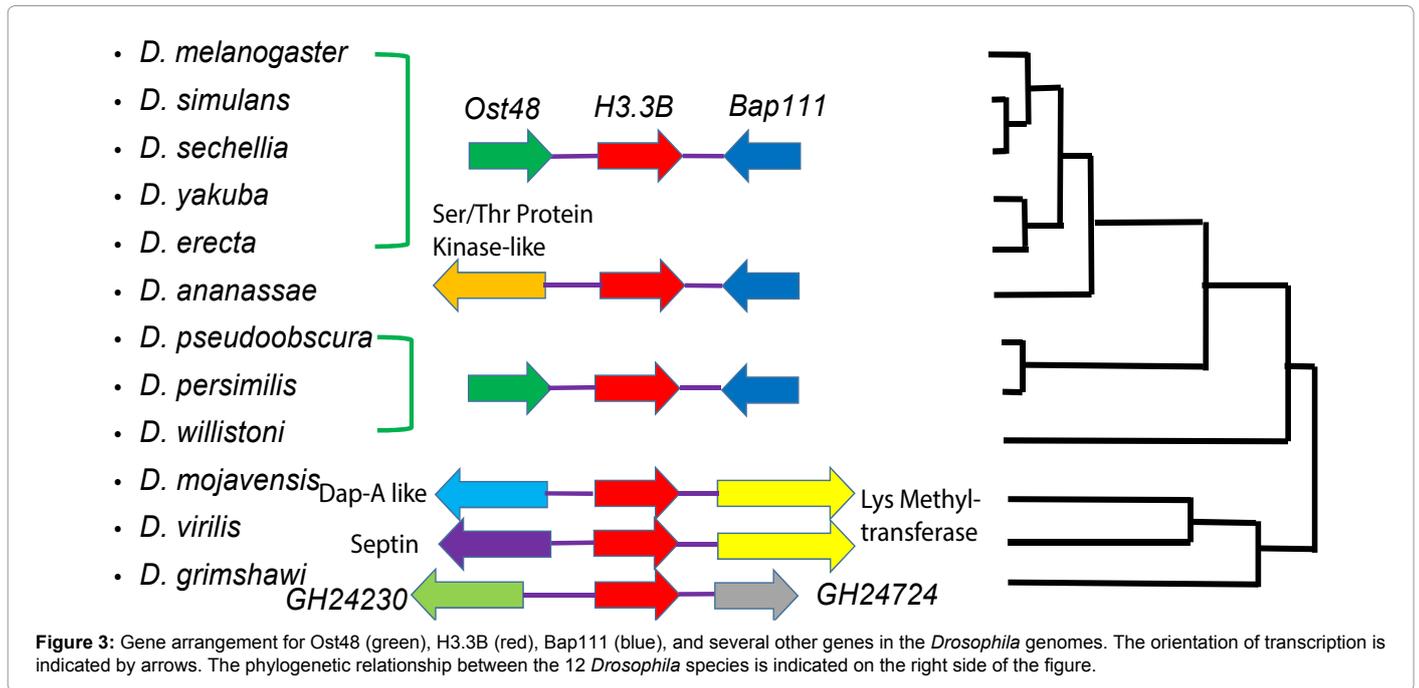
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12 *Drosophila* species are shown diagrammatically in Figures 2 and 3, respectively. For all 12 species, the H3.3A gene was paired with the U11-48K-like gene in a head-to-head manner (Figure 2). The U11-48K-like gene encodes a 48 kDa protein with a zinc finger domain. The strong conservation of pairing of the H3.3A gene and the U11-48K-like gene suggests coordinated transcriptional control. A Rpn11 gene is located downstream of the H3.3A gene in a tail-to-tail configuration in all 12 species. The Rpn11 gene encodes a protein exhibiting thiol-dependent ubiquitin-specific protease activity. However, in five species of the melanogaster group, a peptidase M13 gene has been inserted between the H3.3A gene and the Rpn11 gene.

Five different genes are located upstream of the H3.3B gene and three are located downstream in the 12 *Drosophila* species studied (Figure 3). None of the gene arrangements for the H3.3B gene have been conserved in all 12 *Drosophila* species. A Bap111 gene located downstream of the H3.3B gene was observed in nine species and an Ost48 gene was observed in eight species. The Bap111 gene encodes a DNA binding transcription coactivator with a high mobility group box domain and the Ost48 gene encodes a protein glycosyltransferase 48 kDa subunit. The transcriptional orientation of the Bap111 gene is tail-to-tail relative to the H3.3B gene, suggesting that there is at best a weak relationship between them. However, two genes that are strongly related to histone modification were found in several species: A Ser/Thr





Species	H3 gene	H3.3A gene	H3.3B gene
<i>Drosophila melanogaster</i>	AB019400 (DDBJ)	CG5825 (FlyBase)	CG8989 (FlyBase)
<i>Drosophila simulans</i>	AB019401 (DDBJ)	GD23319 (FlyBase)	GD27984 (FlyBase)
<i>Drosophila sechellia</i>	AB019402 (DDBJ)	GM18523 (FlyBase)	GM13676 (FlyBase)
<i>Drosophila yakuba</i>	AB019405 (DDBJ)	GE11328 (FlyBase)	GE17412 (FlyBase)
<i>Drosophila erecta</i>	AB019406 (DDBJ)	GG25048 (FlyBase)	GG19000 (FlyBase)
<i>Drosophila ananassae</i>	GF21608 (FlyBase)	GF15720 (FlyBase)	GF19171 (FlyBase)
<i>Drosophila pseudoobscura</i>	AB044538 (DDBJ)	GA19158 (FlyBase)	GA22698 (FlyBase)
<i>Drosophila persimilis</i>	AB044539 (DDBJ)	GL26550 (FlyBase)	GL26831 (FlyBase)
<i>Drosophila willistoni</i>	GK19237 (FlyBase)	GK23881 (FlyBase)	GK25162 (FlyBase)
<i>Drosophila mojavensis</i>	GI21876 (FlyBase)	GI16984 (FlyBase)	GI14879 (FlyBase)
<i>Drosophila virilis</i>	GJ18585 (FlyBase)	GJ17231 (FlyBase)	GJ15308 (FlyBase)
<i>Drosophila grimshawi</i>	GH23749 (FlyBase)	GH11489 (FlyBase)	GH24723 (FlyBase)

Table 1: Analysis of the H3, H3.3A and H3.3B genes in 12 *Drosophila* species. Nucleotide sequence data were obtained from FlyBase (annotation ID) or DDBJ (accession number).

protein kinase-like gene was located upstream of the H3.3B gene in *D. ananassae* in a head-to-head orientation, and a *Lys methyltransferase* gene was located downstream of the H3.3B gene in *D. mojavensis* and *D. virilis* with the same transcriptional orientation. It remains unknown whether these gene combinations in these limited species are by random occurrence.

Analysis of the nucleotide sequences outside the coding regions for the H3.3A and H3.3B genes

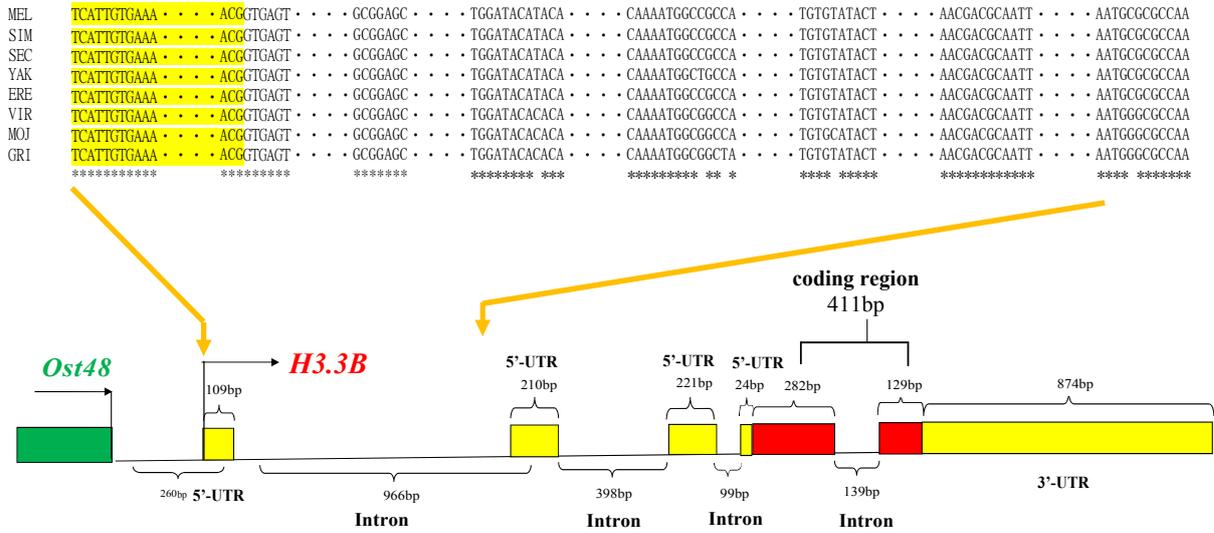
DNA sequences with important roles can be detected by investigating their evolutionary conservation between distantly related

species. The regions upstream and downstream of the H3.3A and H3.3B genes were investigated for the 12 *Drosophila* species. No strongly conserved DNA sequence amongst the 12 species has been identified in the upstream regions of these two genes. Furthermore, no signal sequence for transcription was recognized upstream or downstream of the genes (H3.3A and H3.3B). However, DNA sequences observed for several of the 12 species investigated could have species (or species group)-specific functions. In addition, exons and introns within a gene were studied in the same way as the upstream and downstream regions. Moderately conserved DNA sequences between eight species, including distantly related species, are shown in Figures 4 and 5. Short DNA sequences (7-14 bp) were intermittently preserved in the region of exon 1 and intron 1 for the H3.3B gene (Figure 4). One of the conserved sites corresponded to a splicing site of exon 1. A conserved DNA sequence of about 60 bp outside the protein coding region was recognized in the last exon of the H3.3B gene of eight species (Figure 5). It is possible that small RNA molecules interact with this region. However, a BLAST search with the DNA sequence for the *D. melanogaster* genome failed to detect a similar DNA sequence; consequently, the functional importance of this conserved DNA region of the H3.3B gene should be studied.

Analysis of the coding regions for the H3, H3.3A and H3.3B genes in *Drosophila*

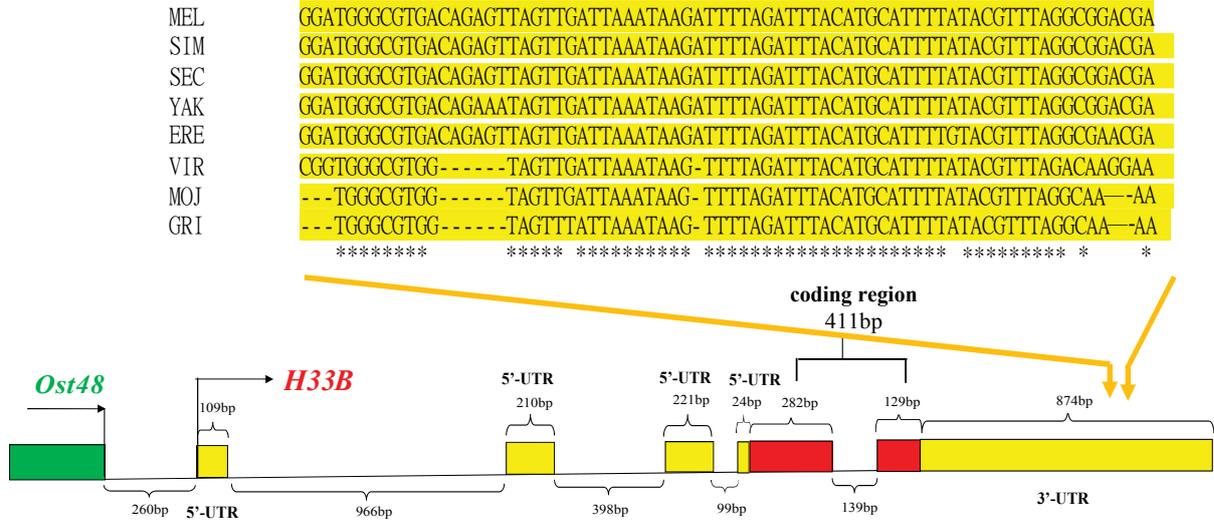
The amino acid sequences of the histones encoded by the H3.3A and H3.3B genes of 12 *Drosophila* species showed no amino acid substitution: all variant H3s in the 12 species (12 H3.3A and 12 H3.3B) had identical amino acid sequences. However, as described previously [15,19-20], four amino acid substitutions (positions 31, 87, 89 and 90) were found between H3 and the H3 variants H3.3A and H3.3B. These substitutions were type-specific, replication-dependent or replacement, but their functional significance remains unknown. A single substitution was found in H3 in three species: at position 28 in *D. pseudoobscura*, at position 7 in *D. erecta*, and at position 124 in *D. persimilis* (Supplementary Figure S).

The codon usage for amino acids was compared for three H3 genes (H3, H3.3A and H3.3B) (Figure 6). Significant differences in codon



Nucleotide sequences of eight *Drosophila* species are aligned. Asterisks under the sequences indicate identical nucleotides at the respective sites in the eight *Drosophila* species. The regions are identified by the same colors as indicated in Figure 1. The second segment corresponds to the splicing site. MEL: *Drosophila melanogaster*, SIM: *Drosophila simulans*, SEC: *Drosophila sechellia*, YAK: *Drosophila yakuba*, ERE: *Drosophila erecta*, VIR: *Drosophila virilis*, MOJ: *Drosophila mojavensis*, GRI: *Drosophila grimshawi*.

Figure 4: Nucleotide sequence comparison of the first exon and the first intron in the *H3.3B* genes of eight *Drosophila* species.



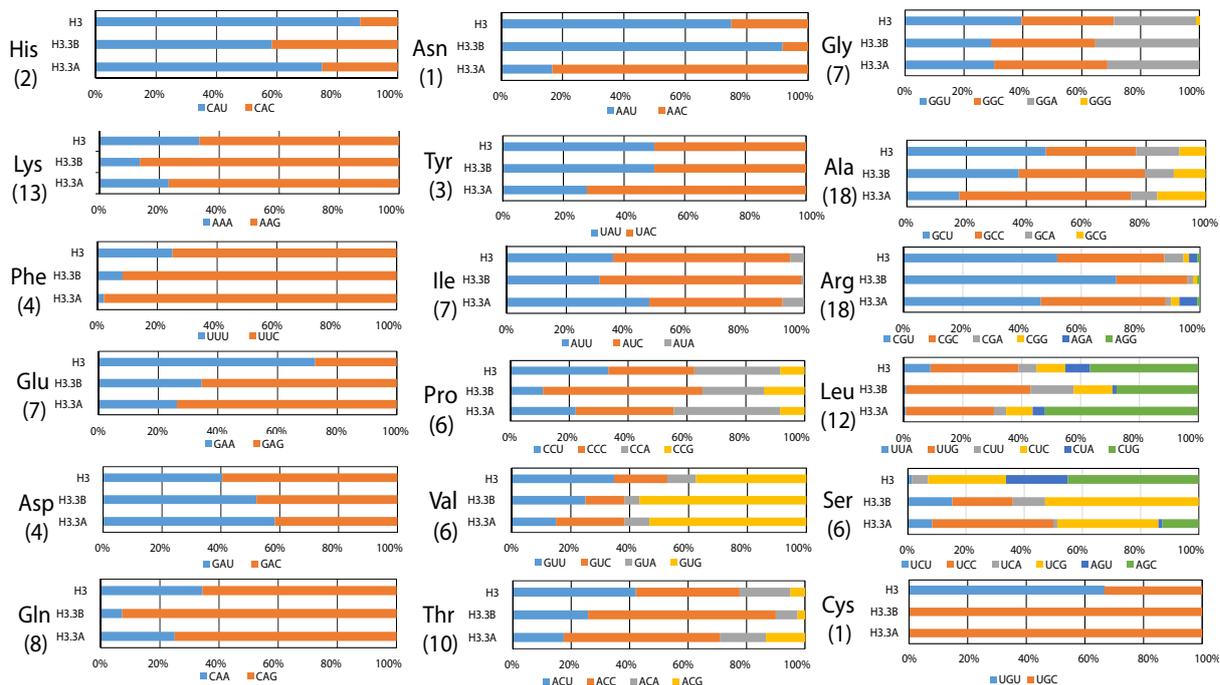
Nucleotide sequences of eight *Drosophila* species are aligned. Asterisks under the sequences indicate identical nucleotides at the respective sites in the eight *Drosophila* species. The regions are identified by the same colors as indicated in Figure 1. MEL: *Drosophila melanogaster*, SIM: *Drosophila simulans*, SEC: *Drosophila sechellia*, YAK: *Drosophila yakuba*, ERE: *Drosophila erecta*, VIR: *Drosophila virilis*, MOJ: *Drosophila mojavensis*, GRI: *Drosophila grimshawi*.

Figure 5: Nucleotide sequence comparison of the fifth exon in the *H3.3B* genes of eight *Drosophila* species.

bias were found for the H3 genes for 13 of 18 amino acids with multiple synonymous codons (Table 2). Of these 13 amino acids, seven (Gln, Asn, Ile, Pro, Ala, Arg, Leu) showed different patterns of codon usage for the H3.3A gene compared to the H3.3B gene, whereas the remaining six amino acids (Glu, Thr, Lys, Ser, Cys, Phe) showed similar patterns of codon usage for both the H3.3A and H3.3B genes. Few codons for Asn and Cys were compared as these amino acids appear only once. The patterns of codon usage were also compared for the H4 and H4r genes; the patterns of codon bias for five amino acids are compared in Figure 7 and the result showed that the pattern of codon usage for H4r was similar to that for H3.3A and H3.3B. For example, AAA was used 33.3% of the time for Lys in the H3 gene and thus AAG was used

66.7% of the time, whereas AAA was used 23.1% and 13.5% of the time in the H3.3A and H3.3B genes, respectively. Thus AAA was used more frequently in the H3 gene compared to the replacement type. A similar tendency was found for the H4 gene: AAA was used 38.6% of the time in H4 but 17.4% of the time in H4r. AAG was used more frequently in the replacement types, H4r, H3.3A and H3.3B genes. These results suggested that for several amino acids, a type-specific (replication-dependent or replacement) pattern was observed for codon usage at certain sites.

Codon usage was analysed site-by-site to study the relationship between codon usage and histone modification (Figure 8). Three



Numbers within the parentheses indicate the number of residues of that amino acid in H3 of *Drosophila melanogaster*.

Figure 6: Codon usage in histone genes for H3, H3.3A and H3.3B in *Drosophila*.

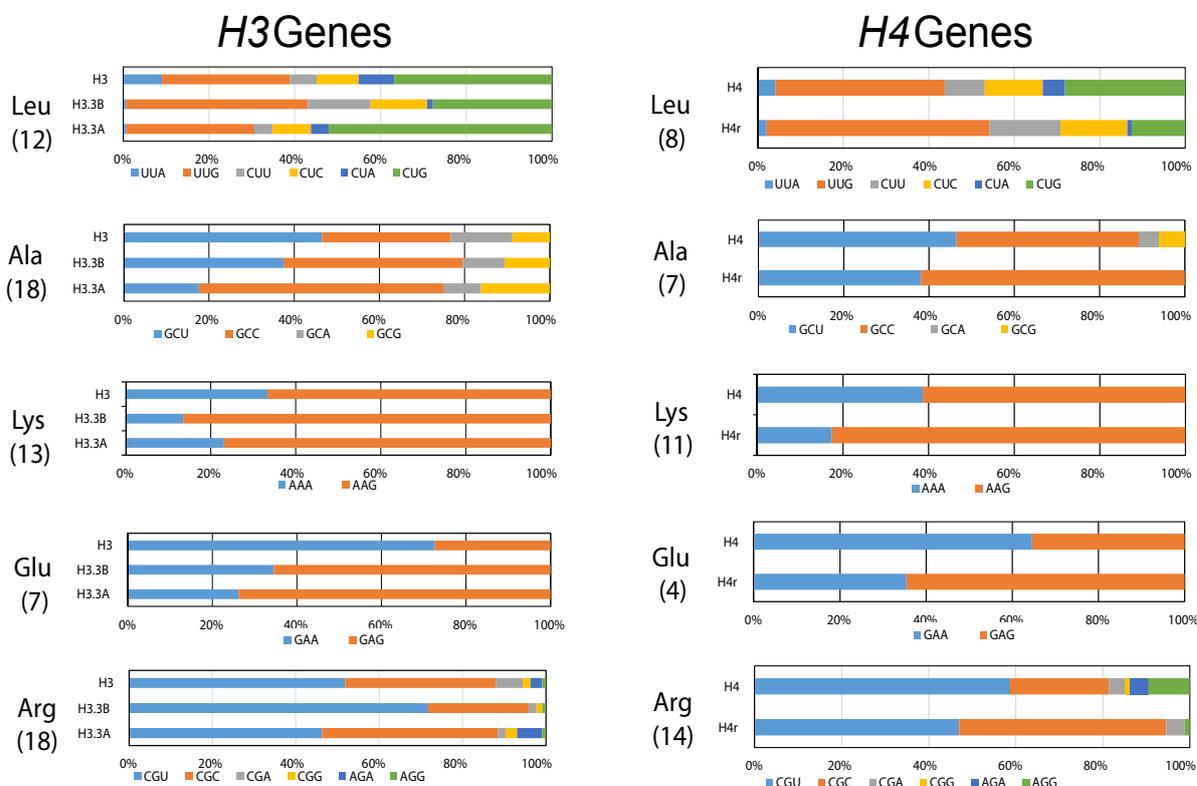


Figure 7: Codon usage comparison for replacement histones and replication-dependent histones of the H3 and H4 genes in *Drosophila*.

histone modifications (methylation, acetylation and phosphorylation) for four amino acids (Lys, Arg, Thr, Ser) were analysed [2,33]. Most of

the sites for Lys (10 of 13 sites) were modified, and some of these sites showed extraordinarily different codon biases. AAG was predominant

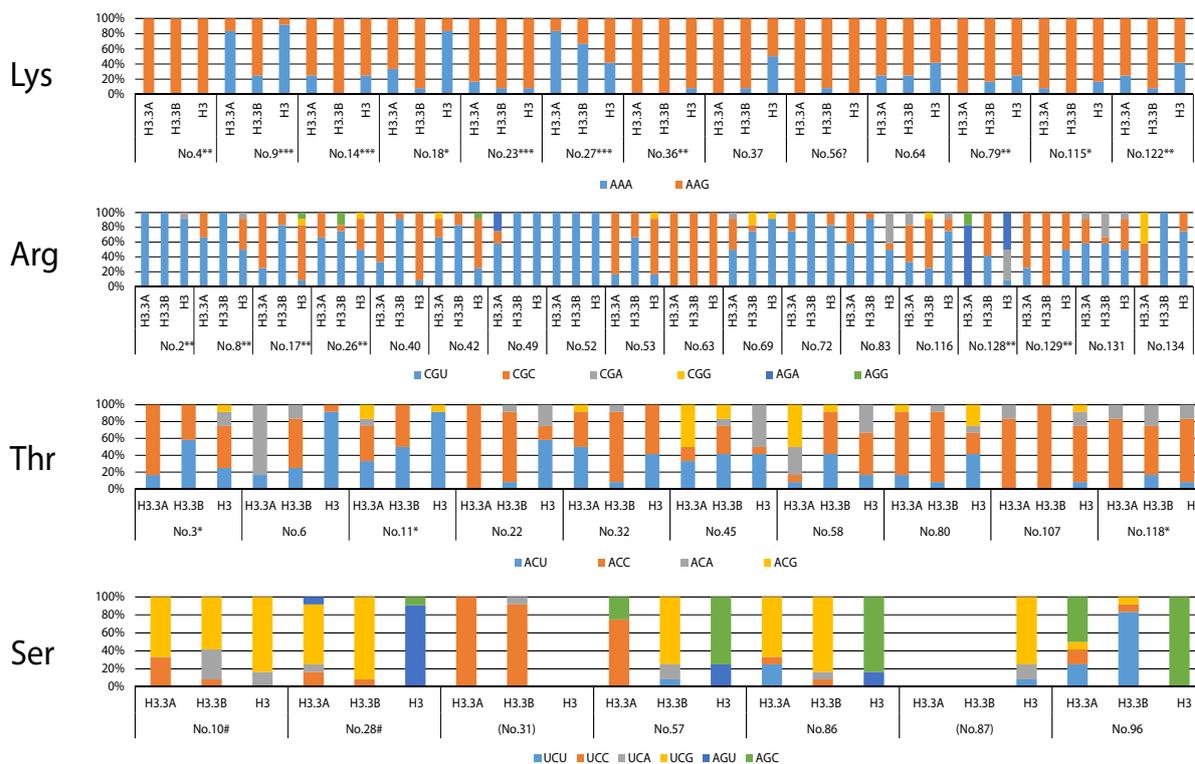


Figure 8: Codon usage at each amino acid site of the respective histone 3 genes in *Drosophila*. The locations of histone modifications are indicated by '(Acetylation), ''(Methylation), ''''(Acetylation and methylation) and #(Phosphorylation) after the position number.

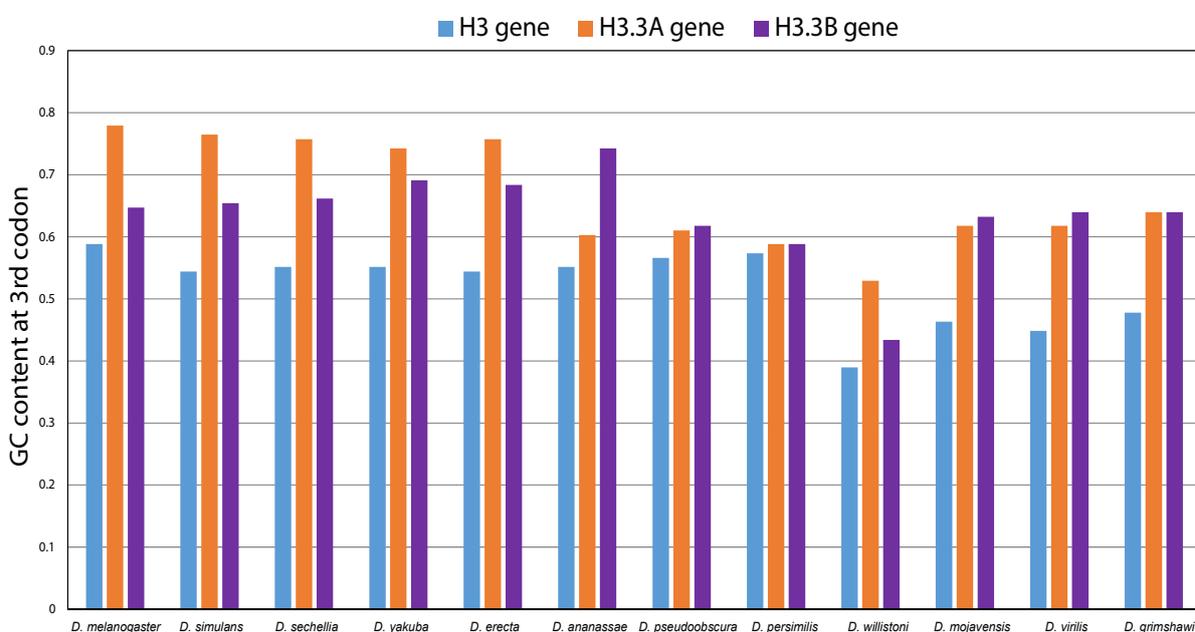


Figure 9: GC content at the 3rd codon positions of the H3 (blue), H3.3A (red) and H3.3B (purple) genes in *Drosophila*.

at positions 4, 36 and 56, whereas AAA was used most frequently at positions 9 and 27 in the replacement types H3.3A and H3.3B. All these sites except 56 were modified, although a recent study suggested that position 56 can be modified [33]. For the Arg sites, CGU was used most of the time at positions 2 and 52, CGC was predominant at positions 17, 40, 53, 63 and 129, and AGA was most frequent at position 128 but

was not used at the other sites. Four of these sites at 2, 17, 128 and 129 were modified sites for Arg. Threonine was more commonly encoded by ACU at position 11 of H3 compared to the other Thr sites. The Ser at position 28 of H3 was typically encoded by AGU and position 28 is one of two phosphorylation sites for Ser. These results indicated that the codon biases observed at several histone modification sites showed

Amino Acid	χ^2	d.f.
Cys	20.57***	2
Thr	39.35***	6
Gly	5.48	6
Arg	47.92***	10
Lys	17.25***	2
Leu	54.23***	10
Ala	55.29***	6
His	5.29	2
Val	10.52	6
Asp	3.13	2
Asn	15.66***	2
Ile	12.57*	4
Gln	21.01***	2
Pro	19.03**	6
Ser	117.50***	10
Tyr	4.85	2
Glu	41.69***	2
Phe	12.94**	2

***P<0.001, **P<0.01, *P<0.05; d.f.: Degree of Freedom

Table 2: χ^2 tests for codon bias in the H3, H3.3A and H3.3B genes in 12 *Drosophila* species.

extraordinary different patterns compared to other sites, suggesting that there is a connection between codon bias and histone modification.

The GC content at the third codon position was compared for the 12 H3 genes (Figure 9). As was found for the H4 genes [12], the GC content at the third codon position was higher in the replacement type genes H3.3A and H3.3B (Figure 9).

Discussion

Analysis of the genomic organization of genes showed that most combinations of gene pairs were not conserved amongst the 12 *Drosophila* species studied. The unique gene pair U-11-48K-like and H3.3A showed a strong connection but strongly conserved DNA sequences for transcriptional signalling or control have not been observed between these genes. The existence of U-11-48K-like and H3.3A genes may result in similar expression conditions such as timing and tissues due to the similar nucleosome structure. Indeed, several functionally related gene pairs, such as a Ser/Thr protein kinase-like gene upstream of the H3.3B gene in *D. ananassae* and a Lys methyltransferase gene downstream of the H3.3B gene in *D. mojavensis* and *D. virilis* were observed for a specific species or species group. If these gene pairs have a biological significance, a narrow distribution in phylogeny suggests that these must be a newly generated or designed gene pair.

Moderately conserved sequences were found within the H3.3B gene, with eight short segments conserved in the first exon and first intron. The significance of these DNA sequences is unknown but they may interact with the spliceosome, and indeed one segment corresponds to the splicing site (Figure 4). A DNA sequence of about 60 bp located outside the coding region at exon 5 in the H3.3B gene is highly conserved and appears to have functional significance. Akhmanova et al. compared the H3.3B genes from *D. melanogaster* and *D. hydei* and also detected these conserved regions [15]. Neither palindrome sequences nor stem-loop structures were detected in this region, indicating that the sequence itself, and not its secondary structure, could interact with small molecules such as RNA. However, similar sequences were not detected in the genome by a BLAST search.

Analysis of the codon bias in H3 genes and the results from H4 genes [12] provided a characteristic bias pattern for several amino

acids in replacement histones, suggesting a connection between translation and histone replacement. Moreover, the 'special' codon bias was observed only for some sites of modified amino acids, further supporting a relationship between translation and histone modification. A better understanding of the 'meaning' of histone modifications may clarify the relationship with codon bias as well as the importance of the translational step in histone replacement, histone modification, and the development of epigenetics.

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