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Genome-wide analyses of two families of snoRNA genes from *Drosophila melanogaster*, demonstrating the extensive utilization of introns for coding of snoRNAs

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ABSTRACT

Small nucleolar RNAs (snoRNAs) are an abundant group of noncoding RNAs mainly involved in the post-transcriptional modifications of rRNAs in eukaryotes. In this study, a large-scale genome-wide analysis of the two major families of snoRNA genes in the fruit fly *Drosophila melanogaster* has been performed using experimental and computational RNomics methods. Two hundred and twelve gene variants, encoding 56 box H/ACA and 63 box C/D snoRNAs, were identified, of which 57 novel snoRNAs have been reported for the first time. These snoRNAs were predicted to guide a total of 147 methylations and pseudouridylations on rRNAs and snRNAs, showing a more comprehensive pattern of rRNA modification in the fruit fly. With the exception of nine, all the snoRNAs identified to date in *D. melanogaster* are intron encoded. Remarkably, the genomic organization of the snoRNAs is characteristic of 8 dUhg genes and 17 intronic gene clusters, demonstrating that distinct organizations dominate the expression of the two families of snoRNAs in the fruit fly. Of the 267 introns in the host genes, more than half have been identified as host introns for coding of snoRNAs. In contrast to mammals, the variation in size of the host introns is mainly due to differences in the number of snoRNAs they contain. These results demonstrate the extensive utilization of introns for coding of snoRNAs in the host genes and shed light on further research of other noncoding RNA genes in the large introns of the *Drosophila* genome.

Keywords: snoRNA; ncRNA; intron; RNA modification; *Drosophila melanogaster*

INTRODUCTION

Small nucleolar RNAs (snoRNAs) represent an abundant group of noncoding RNAs (ncRNAs) mainly involved in rRNA biogenesis (Kiss 2002). With the exception of RNase MRP, all the snoRNAs fall into two major families, box C/D and box H/ACA snoRNAs, on the basis of common sequence motifs and structural features (Balakin et al. 1996). Box C/D snoRNAs share two conserved motifs, the 5' end box C (RUGAUGA) and the 3' end box D (CUGA), whereas the box H/ACA snoRNAs exhibit a common hairpin-hinge-hairpin-tail secondary structure with the H (ANANNA) motif in the hinge region and an ACA triplet 3 nt from the 3' end of the molecule (Ganot et al. 1997).

Several snoRNAs, such as U3, snR30, and RNase MRP, are required for specific cleavage of pre-rRNAs (Kiss 2002). However, the majority of box C/D snoRNAs function as guides for site-specific 2'-O-ribose methylation with most box H/ACA snoRNAs functioning as guides for pseudouridylation in the post-transcriptional processing of rRNAs (Smith and Steitz 1997; Bachellerie et al. 2000). Recent research has shown that some snoRNAs and scaRNAs participate in the modifications of snRNAs (Kiss 2002; Zhou et al. 2002; Tycowski et al. 1998, 2004) and even tRNAs by Archaea homologs (Clouet d'Orval et al. 2001). Moreover, an increasing number of orphan snoRNAs with unknown function have been identified from different eukaryotes, suggesting they play additional roles in cellular processes. The identification of snoRNA homologs in Archaea further demonstrates their ancient origin and preservation during the course of evolution (Bachellerie et al. 2002).

The *Drosophila* rRNAs possess numerous post-transcriptional modifications (Ofengand and Bakin 1997), which has made *D. melanogaster* a good model for studying the expression of

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snoRNA genes (Tycowski and Steitz 2001; Huang et al. 2004). A recent study of experimental Rnomics in the fruit fly identified 66 small ncRNAs, of which 35 species belonged to the two families of snoRNAs (Yuan et al. 2003). Computational analyses of conserved structural and functional motifs have also been applied to identify typical box H/ACA snoRNAs and box C/D snoRNA, resulting in a validation of 10 box H/ACA snoRNAs (Huang et al. 2004) and 26 box C/D snoRNAs from the *Drosophila* genome (Accardo et al. 2004; our unpubl. results). These studies, in addition to other reports (Tycowski and Steitz 2001; Zhou et al. 2002), have identified approximately one-third of the total snoRNAs from *Drosophila* as estimated proportionally to the modifications on rRNAs. Furthermore, some interesting features in the expression of the snoRNA genes have also been observed, such as the discovery of dUhg (Tycowski and Steitz 2001) and intronic box H/ACA snoRNA gene clusters in the fruit fly (Huang et al. 2004). These results encouraged us to perform a large-scale genome-wide analysis of the two families of snoRNAs in *Drosophila* using both experimental and computational Rnomics methods. In an attempt to identify all of the snoRNAs and give an overall view of their genomic organization, we have identified 212 gene variants encoding 119 snoRNAs from the *Drosophila* genome, including 57 novel snoRNAs. This analysis provides more comprehensive data on the pattern of rRNA modifications as well as a panorama of the snoRNA genomic organization in *D. melanogaster*. Moreover, a systematic study on the snoRNA host genes has revealed how extensively and efficiently intron regions have been used for ncRNA coding in the compact genome of the fruit fly.

RESULTS

Identification of 31 novel box C/D snoRNAs from *Drosophila*

A special cDNA library for box C/D snoRNAs was constructed with total *D. melanogaster* RNA. We focused on the cDNA fraction of 60–120 nt according to the size range of most box C/D snoRNAs. After screening to eliminate abundant fragments of rRNAs, 35 box C/D snoRNA variants were identified from the library. Most of the cDNA sequences were satisfactorily intact and exhibited typical features of box C/D snoRNA. Detailed examination of the functional elements of the cDNA sequences revealed 28 box C/D snoRNA species, including 12 novel snoRNAs (Table 1).

We have previously used a eukaryotic box C/D snoRNA search program to identify snoRNA genes in *Oryza sativa* (Chen et al. 2003). This effective computational program was applied in the present study to search the *D. melanogaster* genome for box C/D snoRNAs. The searches were mainly based on primary structural elements of the C/D snoRNAs and the information on rRNA methylation sites in eukaryotes such as mammals and yeast before fruit fly data were available. Computational analysis identified 97 snoRNA candi-

dates that accounted for most of our experimental results and recently reported data (Accardo et al. 2004). Northern blotting was used to verify the novel candidates identified in silico, and 19 novel box C/D snoRNAs were validated (Fig. 1A) and added to our snoRNA database (Table 1). All snoRNA sequences were then used to find their isoforms in the *Drosophila* genome.

In total, 102 gene variants encoding 63 box C/D snoRNAs were identified from the *Drosophila* genome. It has to be pointed out that a scaRNA U85 and three known snoRNAs, snoR684, snoR291, and U3, were not detected in this analysis; however these snoRNAs have been included for further analysis in the discussion. Forty-eight snoRNAs were predicted to guide 54 methylated residues in 5.8S, 18S, and 28S rRNA. Four typical snoRNAs and a scaRNA U85 were guides for internal methylations in U2, U5, and U6 snRNAs. In addition to the guide snoRNAs, 12 snoRNAs with no target in either rRNA and snRNA were termed orphan snoRNAs and their functions remain to be elucidated (Table 1; Supplementary Figure S1).

Identification of 26 novel box H/ACA snoRNAs from *Drosophila*

To study box H/ACA snoRNAs in *Drosophila*, another special cDNA library was constructed with total *D. melanogaster* RNA. In contrast to the box C/D snoRNA research, we focused on the cDNA fraction of 120–180 nt to evaluate the box H/ACA snoRNAs. After screening several thousand cDNA clones to eliminate heavy contamination of rRNA fragments, 31 box H/ACA candidates were identified. Sequence and structural analyses revealed that the candidates represented 29 box H/ACA snoRNA species, including 14 novel species when compared with known snoRNA data (Yuan et al. 2003; Huang et al. 2004).

Based on the conserved secondary structure and functional elements, a computational analysis for other possible box H/ACA snoRNA genes was performed on all the introns of known snoRNA host genes and ribosomal protein genes. Apart from known box H/ACA snoRNAs, 19 novel candidates were identified; 12 of them were further confirmed by the Northern blotting analyses (Fig. 1B) and added to our database.

In total, 110 gene variants encoding 56 box H/ACA snoRNAs were identified from the *Drosophila* genome. When compared with the published data on *Drosophila*, 26 novel box H/ACA snoRNAs have been first reported in this study. Fifty-one snoRNAs were predicted to guide 85 pseudouridylations in 18S and 28S rRNA. Two snoRNAs, Ψ 28S-1232 and Ψ 28S-3186, may guide pseudouridylations for both rRNA and U4 or U5 snRNA. Moreover, five orphan snoRNAs that lack sequence complementary to rRNA and snRNA were also determined (Table 2; Supplementary Figure S2).

TABLE 1. BOX C/D snoRNA genes in *D. melanogaster*

SnoRNA name	Iso	Len (nt)	Exp	Modification	Antisense element	Homology			Location
						Yeast	Plants	Vertebrates	
Me5.8S-G74*	1	71	N. blot	5.8S-Gm74	15 nt (3')		SnoR39bY	Z37	CG13900
Me18S-A28a*(U27)	3	72	+	18S-Am28	13 nt (5')	SnR74(Z4)	U27	U27	DUHG1
Me18S-A425*(Dm425)	1	84	cDNA	28S-A1385	10 nt (5')				CG4863
				18S-Am425	13 nt (3')	SnR52	SnR52Y	U83	
Me18S-C419*	1	70	N. blot	18S-Cm419	13 nt (3')	U14	U14	U14	CG17420
Mel8S-A469*	1	88	N. blot	18S-Am469	13 nt (3')		SnoR17	Z71	CG9253
Me18S-A1061*(DmSnR54)	1	76	+	18S-Am1061	14 nt (5')	SnR54	U59	U59	DUHG5
Me18S-C1096*	1	85	cDNA	18S-C1104	14 nt (5')				CG4863
				18S-Cm1096	11 nt (3')		SnoR20		
Me18S-U1356a*	3	79	cDNA	18S-Um1356	13 nt (3')	SnR55	U33	U33	DUHG8
Me18S-G1358a*	2	75	N. blot	18S-Gm1358	12 nt (5')	SnR40	SnR21	U32	CG33188
Me18S-C1366*	1	100	cDNA	18S-C1366	12 nt (3')				DUHG5
Me18S-A1374*(SnoR122)	1	67	+	18S-Am1374	10 nt (3')				CG31175
Me18S-A1576*(Z5)	1	116	cDNA	18S-Am1576	15 nt (3')				DUHG2
Me18S-G1620*(U25)	1	71	+	18S-Gm1620	11 nt (5')	SnR56	SnR19	U25	DUHG2
Me18S-A1806*	1	78	cDNA	18S-Am1806	12 nt (3')			U82	IR
Me18S-C1831*(SnoR737)	1	90	cDNA	18S-Cm1831	12 nt (5')	SnR70	U43	U43	CG15442
Me18S-G1952*	1	76	N. blot	18S-G1952	10 nt (5')				DUHG5
Me28S-G764*(U21)	1	81	cDNA	28S-Gm764	14 nt (3')			U21	CG8385
Me28S-A771*	1	75	N. blot	28S-A771	11 nt (5')				CG13900
Me28S-A774a*(DmU18)	2	73	+	28S-Am774	12 nt (5')	U18	U18	U18	CG11271
Me28S-C788a*(DmSnR58)	2	70	+	28S-Cm788	15 nt (5')	Z12	SnR58Y	Z12	CG13900
Me28S-G980*	1	71	N. blot	28S-Gm980	11 nt (5')	SnR39b	SnR39bY	SnR39b	CG14792
Me28S-A982a*(DmSnR39/59)	2	77	+	28S-Am982	13 nt (5')	SnR39/59	U51	U51/U32	DUHG4
Me28S-A992*	1	71	N. blot	28S-Am992	12 nt (5')	SnR60	U80	U80/U77	DUHG5
Me28S-G1083a*(DmSnR60)	4	84	cDNA	28S-Gm1083	12 nt (5')	SnR60	U80	U80	DUHG6
				28S-Am1092	10 nt (3')	SnR84	SnoR133		
Me28S-A1322*(Z1)	1	77	cDNA	28S-Am1322	11 nt (5')	SnR61	U38	U38	CG8280
Me28S-A1666a*(U76)	2	76	+	28S-Am1666	13 nt (5')	U24	U24	Z20/U76	DUHG1
Me28S-U1848*	1	80	cDNA	28S-U1848	13 nt (3')				DUHG5
Me28S-C2017*	1	93	cDNA	28S-C2017	11 nt (5')				CG4863
				28S-A3349	15 nt (3')				
Me28S-A2113*	1	73	N. blot	28S-Am2113	14 nt (3')		SnoR33	Z38	CG10652
Me28S-U2134a*(DmSnR62)	2	77	+	28S-Um2134	12 nt (5')	SnR62	U34	U34	CG1475
Me28S-G2173*	1	67	N. blot	28S-Gm2173	12 nt (5')			U50	CG4046
Me28S-A2486*	1	117	N. blot	28S-A2486	12 nt (5')				CG31647
Me28S-A2564*(SnoR442)	1	114	cDNA	28S-Am2564	12 nt (3')	SnR63	U40	U40/U46	CG7808
Me28S-A2589a*	3	106	N. blot	28S-Am2589	12 nt (3')	SnR13	U15	U15	CG9696
Me28S-A2634a*	3	73	cDNA	28S-Am2634	12 nt (5')		SnoR44	U79(Z22)	DUHG7
Me28S-C2645a* (DmSnR64)	3	75	+	28S-Cm2645	12 nt (5')	SnR64	SnoR44	U74(Z18)	DUHG3
				28S-A2653	10 nt (3')				
Me28S-G2703a*	3	91	cDNA	28S-Gm2703	10 nt (3')	SnR190		Z63	DUHG5
Me28S-G3081a*(U31)	4	72	+	28S-Gm3081	13 nt (5')	SnR67	U31	U31	DUHG1
Me28S-G3113a*(Dm3112)	2	80	cDNA	28S-Gm3113	13 nt (5')			U58	CG13900
Me28S-C3227a*	2	80	cDNA	28S-C3227	16 nt (3')				DUHG5
Me28S-G3253*	1	88	N. blot	28S-Gm3253	16 nt (5')	SnR48	SnoR1		CG3395
Me28S-G3255a*(DmSnR48)	2	85	cDNA	28S-Gm3255	16 nt (5')	SnR48		U60	DUHG4
Me28S-G3277a*(SnR38)	3	80	cDNA	28S-Gm3277	13 nt (5')	SnR38	SnR38Y	SnR38	DUHG1
Me28S-C3341a*(U49)	2	80	cDNA	28S-Cm3341	13 nt (5')		U49	U49	CG6253
Me28S-U3344a*	2	79	cDNA	28S-U3344	13 nt (5')				CG1518
Me28S-C3351*	1	77	N. blot	28S-C3351	13 nt (5')				CG12740
				18S-A613	13 nt (3')				
Me28S-A3407a*(U29)	4	90	cDNA	28S-Am3407	11 nt (5')	SnR71	U29	U29	DUHG1
Me28S-C3420a*	2	91	N. blot	28S-Cm3420	12 nt (5')	SnR73	U35	U35	CG8857
MeU2-C28*(SnoR700)	1	87	+	U2-Cm28	13 nt (3')		SnoR101		CG1518
MeU5-U42*(SnoR755)	1	140	+	U5-Um42	13 nt (5')				CG12316
MeU5-C46▲(U85)	1	315	+	U5-Cm46	13 nt (3')			U85	CG1142
				U5-Ψ47	6 + 6 nt (5')				
MeU6-A47*(Z30)	1	93	N. blot	U6-Am47	11 nt (3')	Z30		Z30	CG1666

(continued)

TABLE 1. Continued

SnoRNA name	Iso	Len (nt)	Exp	Modification	Antisense element	Homology			Location
						Yeast	Plants	Vertebrates	
MeU6-C68*	1	99	N. blot	U6-C68	11 nt (5')				CG2173
DmU3a▲(U3)	2	136	+	rRNA cleave		U3	U3	U3	IR
DmU14a*(U14)	2	84	cDNA	rRNA cleave		U14	U14	U14	DUHG2
DmOr_cdl	1	106	cDNA	Unknown					CG3074
DmOr_cd2*(SnoR461)	1	140	cDNA	Unknown					DUHG4
DmOr_cd3*(SnoR229)	1	152	+	Unknown					CG9888
DmOr_cd4*	1	78	N. blot	Unknown					DUHG5
DmOr_cd5(SnoR185)	1	103	cDNA	Unknown					IR
DmOr_cd6▲(SnoR684)	1	170	+	Unknown					CG10576
DmOr_cd7▲(SnoR291)	1	114	+	Unknown					CG1646
DmOr_cd8*	1	92	cDNA	Unknown					CG7808
DmOr_cd9a*	2	91	N. blot	Unknown					DUHG6
DmOr_cd10*(SnoR284)	1	115	cDNA	Unknown					CG3314
DmOr_cd11a*	3	67	N. blot	Unknown					CG12740
DmOr_cd12*(DmU24)	1	93	+	Unknown					CG12740

Only one isoform (isoform a) and the conserved guiding function(s) are given if the snoRNA has more than one variant. (Iso) numbers of isoforms; (Len) length of the SnoRNA gene; (Exp) expression situation. (cDNA, N. blot) SnoRNA was identified by a cDNA library or Northern blotting analysis in our work. (+) Confirmed expression of snoRNAs in other works (Tycowski and Steitz 2001; Yuan et al. 2003; Accardo et al. 2004). SnoRNAs with asterisks were identified by computer-assisted analysis. Those with black triangles were not detected in our work. SnoRNAs identified in previous studies (Tycowski and Steitz 2001; Zhou et al. 2002; Yuan et al. 2003; Accardo et al. 2004) are indicated by their names in parentheses. In the column location, the protein-coding host genes are denoted by their symbols. (IR) Intergenic region. In the column Modification, a nucleotide with "m" represents the rRNA methylation site that is conserved in *Saccharomyces cerevisiae*, plants, and/or mammals.

Two distinguished organizations dominate the expression of the snoRNAs in *Drosophila*

Following the identification of numerous snoRNAs, their genomic organization and expression strategy were then investigated. A large proportion of the box C/D snoRNAs were clearly located in the introns of protein-coding genes such as ribosomal proteins and proteins associated with snoRNP and transcriptional factors. However, 33 gene variants encoding 16 box C/D snoRNAs in addition to two box H/ACA snoRNAs were found clustered in six chromosomal spots that had no annotation (Fig. 2), and therefore were regarded as intergenic spacers in the *Drosophila* genome. Interestingly, each interval between two individual snoRNA genes was ~150–200 nt, which resembles the situations of the two dUhg genes reported previously (Tycowski and Steitz 2001). By searching typical intron splicing signals (5'GT and 3'AG with branching sequences) that flank each of the snoRNA genes, our computational analysis revealed that all the snoRNAs were indeed intron encoded (Table 3) and it was unlikely that the spliced exons of host genes encoded any protein. Therefore, six new UHG-like genes were identified and designated as dUhg 3–8. All six ncRNAs, except dUhg 7, were further supported by the presence of a perfect match with ESTs from the Flybase database (<http://www.flybase.org>, Database of the *Drosophila* Genome) (Fig. 3). As observed in the vertebrate UHG genes (Tycowski et al. 1996), the cDNA transcripts of the dUhg genes in *Drosophila* possessed a poly(A) tail at their 3' end, suggesting products of

RNA polymerase II (Table 3). Together with two previously identified dUhg genes (Tycowski and Steitz 2001), the eight dUhgs encode 53 snoRNA isoforms that represent half of the known box C/D snoRNA genes in *Drosophila*. In contrast to other eukaryotes, dUhg therefore represents a major gene organization for the expression of box C/D snoRNAs in the fruit fly. Of particular interest is that although each dUhg encodes multiple box C/D snoRNAs, the mode of one snoRNA per intron is strictly maintained.

Apart from intron-encoded snoRNAs, three box C/D snoRNAs, including U3 snoRNA, which is involved in rRNA processing, are transcribed independently from intergenic regions, showing a diverse gene organization. It is also worth noting that the U3 snoRNA gene is clustered with a tRNA^{Leu} gene in an inverted repeat (Fig. 4).

In terms of the box H/ACA snoRNAs, most of them are intron-encoded in protein-coding genes, especially rich in ribosomal proteins. A distinguishing characteristic of the genomic organization for this family of snoRNA is the prevalence of intronic clusters. In addition to the clusters reported previously (Huang et al. 2004), a total of 17 intronic clusters encoding 77 box H/ACA snoRNAs (70% of the gene variants identified) have been identified from the *Drosophila* genome (Fig. 4), highlighting the importance of this gene organization for the expression of box H/ACA snoRNAs. In general, the clusters are composed of isoforms of the same snoRNA genes, suggesting that local duplication has served as a major way to form the multiple snoRNA clusters in the introns of protein-coding genes. Interestingly, DmOr-aca4, an

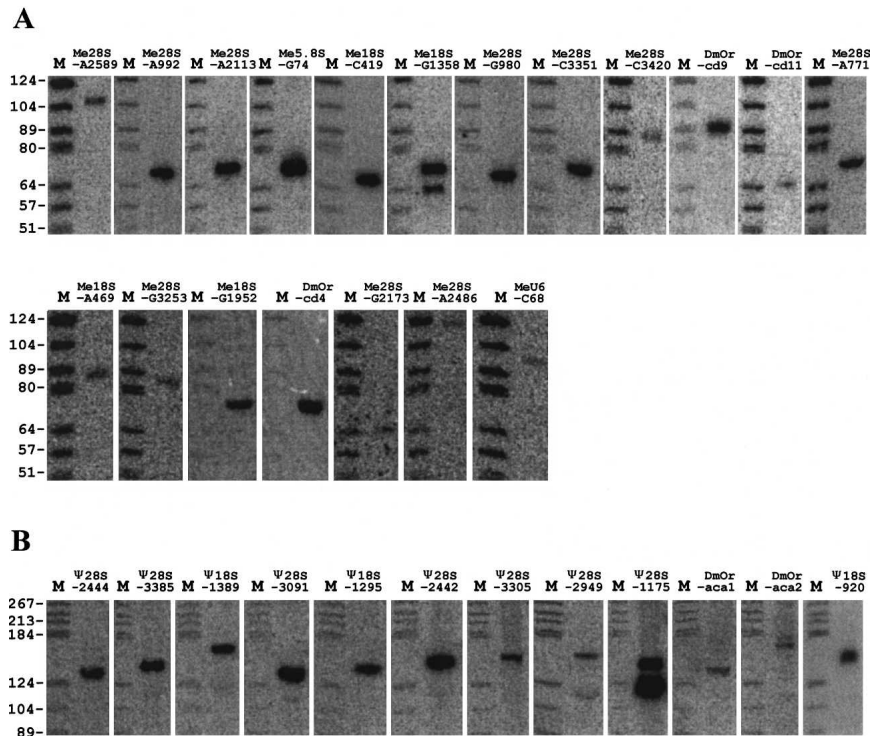


FIGURE 1. Northern blot analyses. Aliquots of 30 μ g total cellular RNA were separated on a denaturing 8% polyacrylamide gel and hybridized with the labeled oligonucleotide probes described in Materials and Methods. (Lane M) molecular weight markers (pBR322 digested with HaeIII and 5'-end labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$). (A) Northern blot analyses of novel box C/D snoRNAs. (B) Northern blot analyses of novel box H/ACA snoRNAs.

orphan snoRNA, was mapped entirely to an intron of a hypothetical pre-mRNA, but was transcribed in the opposite orientation of the protein-coding gene. This peculiar gene organization may suggest a possible antisense function of the orphan snoRNA in the regulation of pre-mRNA processing. In addition to being intron encoded, five box H/ACA snoRNAs appear to be transcribed independently from intergenic regions.

Sixty host genes encoding 207 snoRNAs were scattered over the two large autosomes and the X chromosome of *Drosophila*, but none was found in chromosome 4, which is the shortest chromosome. All the host genes were mapped in the region of euchromatin, with the exception of two located in the heterochromatin where the expression of most genes is not active (Fig. 2). The overall genome-wide analysis of snoRNA genes in *Drosophila* shows a preferential transcription of polycistronic snoRNA from two distinct gene organizations, first, dUhg, which mainly encode box C/D snoRNA, and second, intronic clusters, which dominate the expression of box H/ACA snoRNA.

Extensive utilization of introns for snoRNA-coding in the *Drosophila* host genes

It is evident that in *D. melanogaster* snoRNAs can usually be found in more than one intron of a variety of host genes.

Among the 267 introns in the host genes, more than half of them (145 introns) have been identified as host introns for the snoRNAs and a small ncRNA (Dm184). A higher percentage for snoRNA coding in large introns of the host genes can be attained when taking account of 85 “empty” introns that are smaller than 150 nt, the minimum length required to accommodate one box C/D snoRNA. These empty introns can therefore be easily eliminated from the list of potential host introns. For example, nine of the 13 introns of Dom gene are longer than 150 nt, and 8 turn out to encode 15 snoRNAs and a small ncRNA. In splicing factor 3b, there are nine introns longer than 150 nt, all of which are host introns for snoRNAs. Remarkably, the dUhg genes, which are unlikely to encode any protein, seem to be perfectly designed for snoRNA coding. The eight dUhg genes possess 55 introns in total, only two of which are empty (Fig. 3).

Thirty-seven empty introns larger than 150 nt were further examined in detail. Interestingly, 11 of them contained 50–120-nt-long conserved intronic sequences (LCISs) as compared to their counterparts among different species of *Drosophila*, suggesting the presence of putative *cis*-acting elements or possible small ncRNAs other than snoRNAs. LCIS was also observed in the undefined region of some large introns that were hosts for snoRNAs. Further experiments were performed to validate some of these LCISs and two of them were characterized as stable ncRNAs with unknown function (Fig. 5). The remaining 26 introns (about 10% of the total introns in this study) were devoid of any LCIS. However, 15 of the empty introns (57.7%) belonged to the first intron of ribosomal protein genes or other housekeeping genes. This suggests that instead of snoRNA coding, these introns may have a specialist function, as it has been shown that the first introns that contain binding sites for transcriptional factors are important for the expression of ribosomal protein genes in mammals (Antoine and Kiefer 1998).

The average size of the empty introns was \sim 60–70 bp (Fig. 6), which corresponds perfectly to the length of the minimal intron (61 bp) in *Drosophila* (Yu et al. 2002). Due to snoRNA or snoRNA cluster containing, the host introns can vary in length from 150 bp to 2 kb. However, after removing all snoRNA/snoRNA clusters and ncRNA-coding regions from the 145 host introns, the size distribution of the remaining sequence in these introns was reduced to 90–120 bp, which was slightly longer than the minimal intron length (Fig. 6). Taking account of sequences necessary for

TABLE 2. Box H/ACA snoRNA genes in *D. melanogaster*

SnoRNA name	Iso	Len (nt)	Exp	Modification	Antisense element	Homology			Location
						Yeast	Plants	Vertebrates	
Ψ 18S-110*	1	139	cDNA	18S-Ψ110	6 + 6 nt (3')				CG13900
Ψ 18S-176(SnoR314)	1	154	cDNA	18S-U176	3 + 6 nt (5')				IR
Ψ 18S-301	1	138	cDNA	18S-U301	7 + 5 nt (5')				IR
				18S-Ψ363	4 + 5 nt (3')		SnoR86	U71	
Ψ 18S-525a*	10	134	cDNA	18S-U525	6 + 4 nt (5')				CG10954
				18S-U1313	3 + 10 nt (3')				
Ψ 18S-531*	1	152	N. blot	18S-Ψ531	5 + 10 nt (5')			ACA42	CG7434
				28S-Ψ1850	5 + 7 nt (3')				
Ψ 18S-640a*	7	132	cDNA	18S-Ψ640	6 + 4 nt (5')	SnR161	SnoR73	MBI-12	CG10954
Ψ 18S-841a*(SnoR66)	4	144	+	18S-Ψ841	6 + 6 nt (5')			MBI-13/ACA28	CG9696
Ψ 18S-920*	1	141	N. blot	18S-U920	6 + 5 nt (5')				CG7283
Ψ 18S-996*	1	153	cDNA	18S-Ψ996	5 + 8 nt (3')			ACA14	CG7008
Ψ 18S-1086*	1	147	cDNA	18S-Ψ1086	6 + 4 nt (5')	SnR31	SnoR72	ACA8	CG13900
				28S-U2045	3 + 8 nt (3')				
Ψ 18S-1275*(SnoR783)	1	140	+	18S-Ψ1275	7 + 7 nt (5')	SnR36		ACA36	CG8495
Ψ 18S-1295*	1	140	N. blot	18S-U1295	6 + 3 nt (5')				CG15693
				18S-U740	3 + 8 nt (3')				
Ψ 18S-1347a*(SnoR203)	3	137	cDNA	18S-Ψ1347	7 + 5 nt (5')				CG5119
Ψ 18S-1377a*(SnoR328)	5	139	cDNA	18S-Ψ1279	6 + 6 nt (5')	SnR35		ACA13	CG1883
				18S-Ψ1377	10 + 3 nt (3')	SnR83		ACA4	
Ψ 18S-1389a*	2	159	N. blot	18S-U1389	6 + 7 nt (3')				CG9696
Ψ 18S-1397*	1	141	cDNA	18S-Ψ1397	4 + 7 nt (5')			ACA15	CG10576
			N. blot						
Ψ 18S-1820*(SnoR639)	1	144	cDNA	18S-Ψ1820	7 + 3 nt (3')			U70	CG3333
Ψ 18S-1854a*	3	139	cDNA	18S-Ψ1854	6 + 7 nt (5')				CG8922
			N. blot	18S-Ψ1937	7 + 4 nt (3')				
Ψ 28S-291*(SnoR50)	1	220	cDNA	28S-U291	5 + 8 nt (5')				CG4863
				28S-U2238	4 + 8 nt (3')				
Ψ 28S-1060*	1	150	N. blot	28S-Ψ1060	4 + 7 nt (5')				CG3203
Ψ 28S-1135a*	6	148	N. blot	28S-Ψ1135	6 + 5 nt (3')	SnR8		ACA56	CG8922
Ψ 28S-1153	1	148	cDNA	28S-Ψ1153	7 + 5 nt (5')				IR
				18S-U1425	9 + 6 nt (3')				
Ψ 28S-1175a*	3	136	N. blot	28S-Ψ1175	6 + 6 nt (5')				CG4046
				28S-Ψ1311	6 + 3 nt (3')			ACA32	
Ψ 28S-1180*(SnoR535)	1	145	+	28S-U1930	5 + 8 nt (5')				CG10897
				28S-Ψ1180	12 + 4 nt (3')				
Ψ 28S-1192a*	4	140	N. blot	28S-Ψ1192	7 + 6 nt (5')	SnR5	SnoR81	MBI-20/ACA52	CG8922
Ψ 28S-1232*	1	141	N. blot	U5-U82	7 + 5 nt (5')				CG3203
				28S-U1232	5 + 4 nt (3')				
Ψ 28S-1837a*(SnoR14)	3	137	cDNA	28S-Ψ1837	6 + 7 nt (5')				CG10576
Ψ 28S-2149*(SnoR143)	1	144	+	28S-U2149	7 + 6 nt (5')				CG1883
				28S-U18	4 + 7 nt (3')				
Ψ 28S-2442a*	2	140	N. blot	28S-Ψ2442	6 + 3 nt (5')	SnR3		ACA6	CG2922
				28S-Ψ2533	8 + 6 nt (3')				
Ψ 28S-2444*	1	130	N. blot	28S-Ψ2444	7 + 4 nt (5')		SnoR87	ACA19	CG7726
Ψ 28S-2562*	1	135	cDNA	28S-U925	6 + 4 nt (5')				CG15693
				28S-Ψ2562	7 + 6 nt (3')		SnoR92	MBI-1/ACA23	
Ψ 28S-2566*(SnoR269)	1	268	+	28S-Ψ2566	5 + 5 nt (5')	SnR191	SnoR79	U19/MBI-26	CG3902
				28S-Ψ2568	7 + 6 nt (3')	SnR191	U19	U19	
Ψ 28S-2622(SnoR3)	1	140	cDNA	28S-U363	3 + 7 nt (5')				IR
				28S-Ψ2622	5 + 6 nt (3')		SnoR83	ACA48	
Ψ 28S-2626*	1	142	N. blot	28S-Ψ2626	6 + 4 nt (3')				CG1883
Ψ 28S-2648*(SnoR72)	1	162	+	28S-Ψ2648	5 + 4 nt (5')	SnR9		MBI-12/ACA58	CG7424
Ψ 28S-2719*(SnoR734)	1	145	cDNA	28S-Ψ2719	6 + 3 nt (5')				CG7434
Ψ 28S-2876*(SnoR825)	1	140	+	28S-Ψ2956	4 + 7 nt (5')				CG8922
				28S-U2876	9 + 3 nt (3')				
Ψ 28S-2949*	1	155	N. blot	28S-Ψ2949	5 + 5 nt (5')				DUHG4
Ψ 28S-2996*	1	136	cDNA	28S-U2996	5 + 8 nt (5')				CG17489
Ψ 28S-3091a*	2	135	N. blot	28S-Ψ3091	7 + 4 nt (5')		SnoR78		CG2922

(continued)

TABLE 2. Continued

SnoRNA name	Iso	Len (nt)	Exp	Modification	Antisense element	Homology			
						Yeast	Plants	Vertebrates	Location
Ψ 28S-3186*(SnoR165)	1	143	cDNA	28S-Ψ3356	8 + 6 nt (3')			ACAI	CG2922
				U4-U59	7 + 4 nt (5')				
				28S-Ψ3186	5 + 6 nt (3')				
Ψ 28S-3305a*	3	150	N. blot	28S-Ψ3305	5 + 4 nt (3')			E3	CG9983
Ψ 28S-3308*	1	153	cDNA	28S-Ψ3308	9 + 4 nt (3')			U68	CG13900
Ψ 28S-3316a*	5	132	N. blot	28S-Ψ3316	6 + 3 nt (5')			MBI-3/ACA21	CG9696
Ψ 28S-3327a*(SnoR586)	3	140	cDNA	28S-Ψ3327	6 + 6 nt (5')	SnR46		ACA16	CG11276
				18S-U1920	4 + 6 nt (3')				
Ψ 28S-3342(SnoR644)	1	164	cDNA	28S-Ψ3342	7 + 3 nt (3')	SnR34	U65	U65	IR
Ψ 28S-3378*	1	150	N. blot	28S-Ψ3378	4 + 8 nt (5')				CG9696
Ψ 28S-3385a*	2	150	N. blot	28S-Ψ3385	6 + 4 nt (3')	SnR10	SnR74	MBI-3/ACA21	CG2922
Ψ 28S-3405a*	4	140	cDNA	28S-Ψ3405	7 + 5 nt (5')	SnR37		ACA10	CG9983
				28S-U1265	7 + 5 nt (3')				
Ψ 28S-3436a*(SnoR708)	2	142	cDNA	28S-Ψ3436	6 + 5 nt (5')	SnR42		MBI-6/ACA27	CG3203
Ψ 28S-3571*	1	144	cDNA	28S-Ψ3571	5 + 5 nt (3')				CG2922
				18S-U1790	5 + 7 nt (5')				
DmOr_aca1*	1	135	N. blot	Unknown					CG4046
DmOr_aca2*	1	142	N. blot	Unknown					CG10944
DmOr_aca3*	1	145	cDNA	Unknown					CG5502
DmOr_aca4	1	162	cDNA	Unknown					Intron antisense
DmOr_aca5*(SnoR227)	1	148	cDNA	Unknown					DUHG4

Only one isoform (Isoform a) and the conserved guiding function(s) are given if the snoRNA have more than one variant (Iso) numbers of Isoforms; (Len) length of the snoRNA gene; (Exp) expression situation. (cDNA, N. blot) SnoRNA was identified by a cDNA library or Northern blotting analysis in our work. (+) Confirmed expression of snoRNAs in other work (Yuan et al. 2003). SnoRNAs with stars were identified by computer-assisted analysis. SnoRNAs identified in a previous study (Yuan et al. 2003) are indicated by their names in parentheses. In the column Location, the protein-coding host genes are denoted by their symbols. (IR) Intergenic region. In the column Modification, Ψ represents rRNA pseudouridine sites that are conserved in *Saccharomyces cerevisiae* and/or mammals, and the known rRNA pseudouridines determined. (U) Predicted pseudouridine site that has not been confirmed experimentally.

snoRNA processing (Hirose and Steitz 2001), the intron hosts are only sufficiently long enough to hold a snoRNA gene or gene cluster without any redundant sequence. Interestingly, this compact structure of the host introns appears strictly maintained to encode the two families of snoRNAs in different host genes. In fact, this compact structure of host introns results mainly from parsimonious spacers from the snoRNA-coding region to the 5' splice site, most of which were centered around 30–40 bp despite the intron sizes varying to a large extent (Fig. 7). The distance from the snoRNA gene/gene cluster to the 3' splice sites averaged between 60 and 80 bp, which was very similar to the intronic positioning of box C/D snoRNA genes in mammals. This distance has been proven to be important for the effective processing of the snoRNAs from their host mRNA precursor (Hirose and Steitz 2001).

DISCUSSION

A more complete list of the two families of snoRNA genes in *Drosophila*

To obtain a comprehensive understanding of the genomic organization and expression strategy of ncRNA in

D. melanogaster, we have performed a large-scale analysis of the two major families of snoRNA genes using both experimental and computational RNomics methods. In this study, 212 gene variants encoding 56 box H/ACA and 63 box C/D snoRNAs have been identified in the fruit fly. These data are consistent with previous works (Tycowski and Steitz 2001; Yuan et al. 2003; Accardo et al. 2004; Huang et al. 2004) and further includes 57 novel snoRNAs. In addition, two novel small ncRNAs other than snoRNAs were also validated. This extensive study indicates the complexity of the snoRNA gene families in the *Drosophila* genome and, furthermore, strengthens the analytical strategies used in this research, such as size-fractionated cloning for specialized cDNA libraries that are enriched in snoRNAs. It is also evident that the computational analysis of the *Drosophila* genome is complementary to the cDNA cloning approach. In *D. melanogaster*, a high degree of pseudouridylation on rRNA has been reported (Ofengand and Bakin 1997), while rRNA methylation sites have not yet been mapped. In this study, we have added to the long list a number of novel box C/D snoRNAs that are absent from previous works (Tycowski and Steitz 2001; Yuan et al. 2003; Accardo et al. 2004), reflecting that the level of rRNA methylation in *Drosophila* is not low. According to our

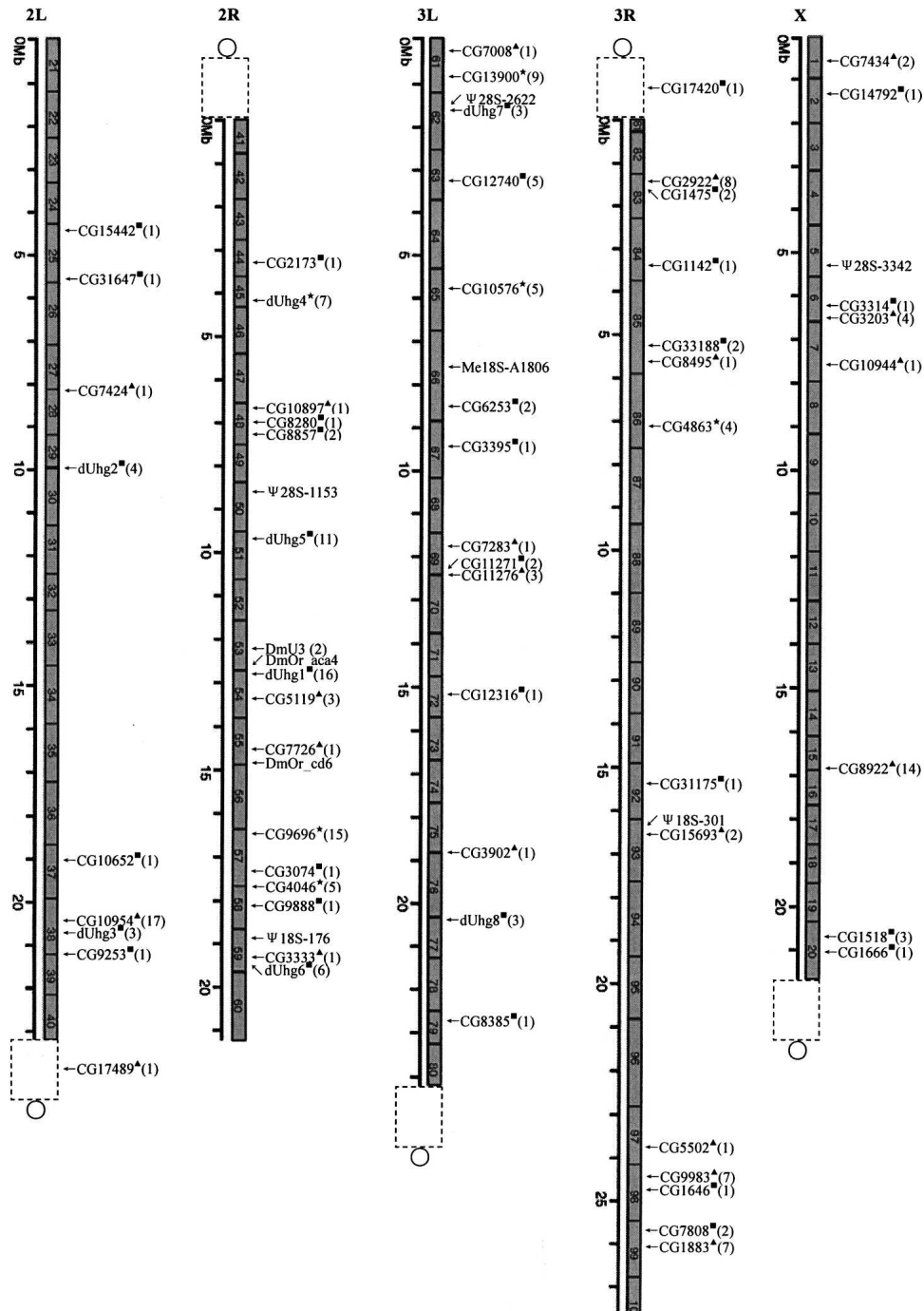


FIGURE 2. Chromosomal mapping of snoRNA genes in *D. melanogaster*. The gray and the empty boxes represent euchromatin and heterochromatin, respectively. Centromere is denoted by an empty circle. Intronic snoRNA gene/gene clusters are indicated by their host genes on the map. The black triangle, box, and asterisk indicate host genes encoding box H/ACA snoRNA only, box C/D snoRNA only, and both of them, respectively. The number in parentheses shows the sum of the snoRNAs encoded by the host genes.

computational analysis of the *Drosophila* genome, we have further predicted that the number of box C/D snoRNA for rRNA is about 97, although dozens of them remain to be confirmed by experimental detection. Interestingly, most of the box C/D snoRNAs from *Drosophila* possess only one antisense sequence, which is predicted to guide a

single methylation site in the RNAs. In contrast, more than half of the box H/ACA snoRNAs exhibit two functional elements guiding two pseudouridylations in the RNAs (some of which are predicted to guide two pseudouridylations in rRNA and snRNA). It is not clear whether the high proportion of the bifunctional box H/ACA

TABLE 3. Eight dUhg host genes in *D. melanogaster*

Name	Chr	Int.	Int. for sno.	Splice signal		EST record	Poly(A) tail	Accession no.
				5' end	3' end			
dUhg1	2R	16	16	GTAAG (7), GTAAT (3) GTGTG (2), GTGGG (1) GTTAG (1), GTAGG (1) GTATG (1)	ACAG (6), GCAG (4) TTAG (2), CCAG (2) ATAG (1), TCAG (1)	*	+	*
dUhg2	2L	4	4	GTAAG (2), GTAAT (2)	GCAG (2), CCAG (2)	*	+	*
dUhg3	2L	4	3	GTAAG (4)	TTAG (2), GCAG (1) ACAG (1)	SD21194 GH14469	+	AY805214
dUhg4	2R	7	7	GTAAG (3), GTAAT (2) GTAAG (1), GTTAG (1)	GCAG (3), TCAG (3) ACAG (1)	EN01530	n.d.	AY805215
dUhg5	2R	11	11	GTAAG (4), GTAAT (2) GTATG (1), GTGAG (1) GTAGG (1), GTAAG (1) GTATA (1)	ACAG (4), GCAG (2) TTAG (2), TCAG (1) CTAG (1), AAAG (1)	SD22445	+	AY805219
dUhg6	2R	6	6	GTAAG (3), GTAAG (1) GTTAG (1), GTGAG (1)	ACAG (3), TCAG (1) GCAG (1), GTAG (1)	EK133426 EP02059	n.d.	AY805216
dUhg7	3L	3	3	GTGTG (2), GTAAT (1)	TCAG (2), ACAG (1)	n.d.	n.d.	AY805217
dUhg8	3L	4	3	GTAAG (1), GTAAT (1) GTAA (1), GTATT (1)	ACAG (2), TCAG (1) GCAG (1)	CK01480	n.d.	AY805218

(Int) number of introns in a host gene; (Int. for sno) number of introns encoding for snoRNA. Number in the parentheses indicates the frequency of donor or acceptor sequences found in introns; (n.d.) situation is unknown; (*) dUhg1 and dUhg2 that had been experimentally verified in previous work (Tycowski and Steitz 2001).

snoRNAs is relative to the constraint of high rRNA pseudouridylation in *Drosophila* or to a selective advantage in the functional evolution of these snoRNAs. From the distribution of the modified residues predicted by the snoRNAs, we estimate that more than two-thirds of the guide snoRNA for rRNA methylation and pseudouridylation in *Drosophila* have been described in this study. Although by no means exhaustive, the analysis has provided a more complete list of the two families of snoRNA genes in *Drosophila*.

Evolution of snoRNA gene organization and expression strategy

Identification of a large number of snoRNA genes provides a unique opportunity to investigate the genomic organization and expression of the ncRNAs in *Drosophila*. Similar to vertebrates (Maxwell and Fournier 1995), almost all snoRNAs in *Drosophila* are intron encoded. This organization not only emphasizes the utility of introns that have long been considered as junk DNA (Flam 1994), but also suggests an intriguing link between mRNA splicing and the expression of snoRNAs that are mainly involved in the post-transcriptional modifications of rRNAs in higher animals. In general, the host genes of snoRNAs are mostly protein-coding genes related to ribosomal biogenesis and nucleolar formation, showing expressional coordination among the various components of the protein translation

machinery (Bachelier et al. 2000). Some ncRNA genes, such as UHG, have also served as host genes specifically encoding box C/D snoRNA, which demonstrates introns, instead of exons, in an RNA precursor producing stable and functional RNAs. Since UHG was originally identified in mammals (Tycowski et al. 1996), only a few UHG-like genes have been further identified for snoRNA coding. In this study, we have shown at least eight dUhgs in the *Drosophila* genome. The abundance of these genes together with their powerful ability for snoRNA coding is a characteristic of snoRNA gene organization in *Drosophila*. Thus the importance of large ncRNAs, such as UHG, for small RNA coding, particularly in higher animals, may be underestimated because most of the noncoding genes in the mammal genome remain to be annotated (News Staff 2004). For instance, recent studies have revealed that some imprinted ncRNA genes in mammals comprise a large number of repeated tandem introns that encode box C/D snoRNAs specifically expressed in brain (Cavaille et al. 2000, 2002). It remains to be answered why in both mammals and *Drosophila*, the UHG or dUhg, respectively, encode mainly box C/D snoRNAs, but rarely box H/ACA snoRNAs. For example, the eight dUhgs in *Drosophila* contained only two box H/ACA snoRNAs compared to 51 box C/D snoRNAs.

The majority of box H/ACA snoRNAs are also intron encoded in *Drosophila*, but they favor another gene organization, that is, intronic cluster. SnoRNA gene clusters were originally found in higher plants (Leader et al. 1995) and

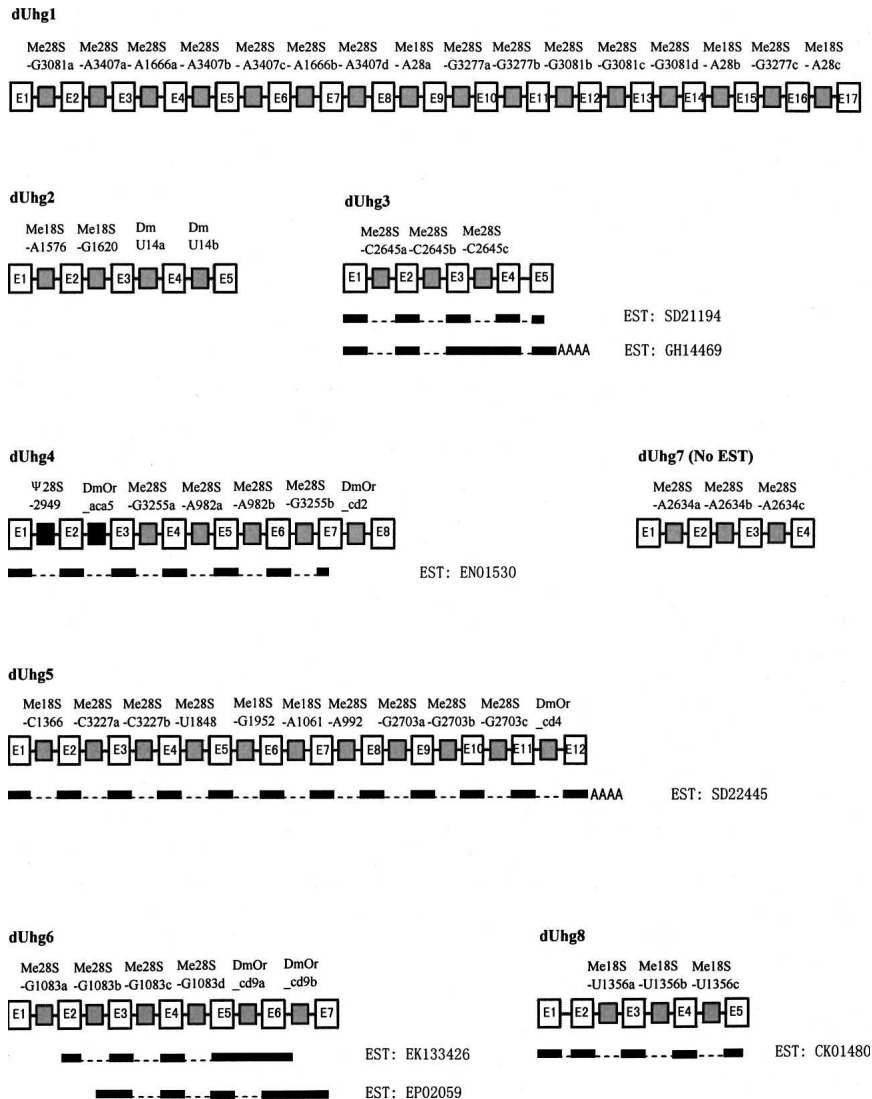


FIGURE 3. Schematic diagram of eight dUhg genes in *D. melanogaster*. Exons and introns of host genes are indicated by empty boxes and solid lines, respectively. Box H/ACA and box C/D snoRNA genes within introns are indicated by black and gray boxes, respectively. Names of host genes and snoRNA genes are also shown. The black bars indicate the cDNA and the spliced introns are indicated by dashed lines. The EST ID is also shown.

the budding yeast *Saccharomyces cerevisiae* in which most snoRNAs are independently transcribed from singletons and five polycistronic snoRNA clusters (Lowe and Eddy 1999; Qu et al. 1999). Gene clusters have also been found in some primordial organisms, such as trypanosomes (Dunbar et al. 2000), *Euglena* (Russell et al. 2004) and *Giardia* (our unpubl. results), implying that the clusters may be an ancient gene organization conserved through the course of evolution. Remarkably, both intronic and independently transcribed polycistrons have considerably developed and become the predominant genomic organizations of snoRNAs in flowering plants (Qu et al. 2001; Liang et al. 2002; Brown et al. 2003; Chen et al. 2003). An atypical discistron, the tRNA-snoRNA

gene cluster, was also identified in the rice genome (Kruszka et al. 2003). The intronic polycistron has never been reported in metazoan other than *Drosophila* and recognized as a predominant genomic organization for the box H/ACA snoRNAs.

Among all the organisms analyzed so far, it is only in *Drosophila* that the two families of snoRNAs exhibit such an important divergence in gene organization and expression strategies. This may reflect intrinsic differences in the mechanisms by which the two families of snoRNA genes evolved respectively during the speciation of the fruit fly.

It is worth noting that box H/ACA snoRNAs have many more isoforms than C/D snoRNAs in *Drosophila*. Unlike C/D snoRNA isoforms, which are highly conserved, mutations occurred frequently in box H/ACA snoRNA isoforms. The conserved secondary structures and box elements in H/ACA isoforms remain unchanged, which guarantees the maturation of snoRNA. Interestingly, the accumulation of mutations in the isoforms would lead to partial alternation of snoRNA's function in loss or gain of rRNA complementary sequences (Supplementary Table S1).

Intronic regions deserve more attention in searching for ncRNAs in *Drosophila*

As a type of intervening sequence of genes, splicing introns with a large size variation are present in most (especially multicellular) eukaryotes (Logsdon 1998; Nixon et al. 2002). By comparison

to the housefly, *Drosophila* possesses a very small genome; however, a large number of introns (about five introns per gene) have been estimated for the *Drosophila* genome (Yu et al. 2002). Interestingly, comparative genomic analysis has revealed similar constraints in the intergenic and intronic sequences of the *Drosophila* genome and about one-fourth of intronic sequences are conserved (Bergman and Kreitman 2001). A draft expression map of the *Drosophila* genome has also shown thousands of uncharacterized transcripts expressed from noncoding DNA in a developmentally coordinated manner (Stolc et al. 2004). Nevertheless, the variation of intron sizes and function of most introns in the *Drosophila* genome still remain to be elucidated.

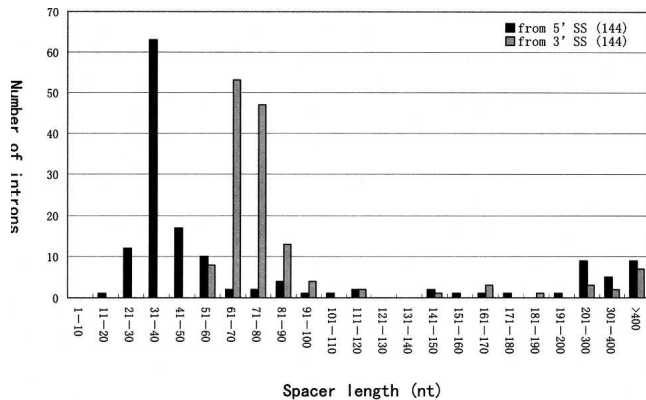


FIGURE 7. Distribution of lengths of spacer sequences for 144 *D. melanogaster* snoRNA genes/gene clusters. The black and gray bars represent distances from 5' and 3' splice sites, respectively.

42°C. The reaction mixture was separated on a denaturing 8% polyacrylamide gel (8 M urea, 1× TBE buffer). cDNAs with sizes ranging from 60 to 120 nt for box C/D cDNA library and ranging from 120 to 180 nt for box H/ACA cDNA library were excised and eluted from the gel. cDNAs were tailed with poly(dG) at the 3' end by using terminal deoxynucleotidyl transferase (Takara), and then amplified by PCR with primers Hin dIII(T)₁₆ and Bam HI(C)₁₆, and cloned into plasmid pTZ18 as described previously (Zhou et al. 2002). The two cDNA libraries were screened by PCR with the P47 and P48 universal primer pair. Only the recombinant plasmids carrying fragments of the expected size were selected for sequencing, which was performed with an automatic DNA sequencer (Applied Biosystems, 377) using the Big Dye Deoxy Terminator cycle-sequencing kit (Applied Biosystems).

An aliquot of 30 µg total RNA was analyzed by electrophoresis on 8% acrylamide/7 M urea gels. Electrotransfer onto nylon membrane (Hybond-N+; Amersham) was followed by UV irradiation for 5 min. Hybridization with 5'-labeled probes was performed as previously described (Zhou et al. 2002).

Oligodeoxynucleotides

Oligonucleotides were synthesized and purified by Sangon Co. The sequences of oligonucleotide probes used for Northern blotting and reverse transcription and oligonucleotide primers used for cDNA libraries construction and screening are shown in Supplementary Table S2. The primers and probes used in reverse transcription and Northern blotting were 5'-end labeled with [γ -³²P] ATP (Yahui Co.) and submitted to purification according to standard laboratory protocols as previously described (Sambrook et al. 1989).

Database accession codes

All novel snoRNA gene and dUHG host gene sequences identified in this study have been deposited in the EMBL and GenBank databases. Accession numbers are shown in Table 3 and Supplementary Table S1.

Supplementary materials

Supplementary materials are available upon request (send an e-mail message containing the keyword "Drosophila snoRNA" to lsbrc04@zsu.edu.cn).

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