A Test of Double Interspecific Introgression of Nucleoporin Genes in Drosophila

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ABSTRACT In interspecific hybrids between Drosophila melanogaster and Drosophila simulans, the D. simulans nucleoporin-encoding Nup96sim and Nup160sim can cause recessive lethality if the hybrid does not also inherit the D. simulans X chromosome. In addition, Nup160sim leads to recessive female sterility in the D. melanogaster genetic background. Here, we conducted carefully controlled crosses to better understand the relationship between Nup96sim and Nup160sim. Nup96sim did not lead to female sterility in the D. melanogaster genetic background, and double introgression of Nup96sim and Nup160sim did not generally lead to lethality when one was heterozygous and the other homozygous (hemizygous). It appears that introgression of additional autosomal D. simulans genes is necessary to cause lethality and that the effect of the introgression is dominant to D. melanogaster alleles. Interestingly, the genetic background affected dominance of Nup96sim, and double introgression carrying homozygous Nup96sim and hemizygous Nup160sim resulted in lethality. Thus, Nup96sim and Nup160sim seem to be two components of the same incompatibility.

KEYWORDS Drosophila hybrid inviability hybrid sterility nucleoporin reproductive isolation speciation

A handful of hybrid incompatibility genes that are responsible for reproductive isolation between species have been identified (Johnson 2010; Presgraves 2010; Maheshwari and Barbash 2011; Ferree and Prasad 2012; Sawamura 2012). Surprisingly, two of these genes in the genus Drosophila encode the nuclear pore proteins (nucleoporins = Nups), which were previously thought to be functionally conserved among diverse organisms. Approximately 30 different Nups assemble to form the nuclear pore complex (NPC) and are essential for nucleocytoplasmic transport, gene regulation, and kinetochore formation (Baptiste et al. 2005; Strambio-De-Castillia et al. 2010; Adams and Wente 2013). Nup96 and Nup160 have been identified as reproductive isolation genes by deficiency mapping in which male hybrids were rescued from the independent lethality by Lethal hybrid rescue (Lhr) mutation of D. simulans. D. melanogaster/D. simulans hybrids carrying the D. simulans Nup96sim and Nup160sim are lethal in hemizygotes (or homozygotes) if they do not inherit the D. simulans X chromosome (Figure 1, A and B), and Nup160sim leads to recessive female sterility in the D. melanogaster genetic background (Presgraves et al. 2003; Tang and Presgraves 2009; Sawamura et al. 2010). Furthermore, positive natural selection and intermolecular coevolution have been demonstrated for several Nup genes including Nup96 and Nup160 in the genus Drosophila (Presgraves and Stephan 2007; Clark and Aquadro 2010; Mensch et al. 2013; Nolte et al. 2013).

Both Nup96 and Nup160 (yeast homologs are Nup145C and Nup120, respectively) are components of the conserved Nup107—160 complex that has a role in the initial assembly of the NPC and functions as a stable anchoring point for other Nups—referred to as central scaffold Nups (Walther et al. 2003; Rasala et al. 2006; Grossman et al. 2012). The Nup107—160 complex forms a Y-shaped structure composed of two short arms—one composed of Nup160 and the other of Nup85—and an extended stalk that is connected to the two arms by Nup96 (Lutzmann et al. 2002; Brohawn et al. 2008; Bilokapic and Schwartz 2012; Szymborska et al. 2013). Because Nup96 and Nup160 interact directly (Leducq et al. 2012), it is reasonable to speculate that the lethality caused by Nup96sim and that caused by Nup160sim in the D. melanogaster/D. simulans hybrids are two distinct
aspects of the same incompatibility. In this context, it is notable that protein–protein interactions between Nup96 and Nup160 are species-specific, as revealed in yeast sibling species and their hybrids (Leduq et al. 2012).

We conducted interspecific crosses of Drosophila to address the following three questions. (1) Does Nup96sim lead to female sterility in the D. melanogaster genetic background as seen with Nup160sim introgression? (2) Does the Nup96sim and Nup160sim double introgression lead to lethality when one is heterozygous and the other homozygous (or hemizygous) in the D. melanogaster background (Figure 1, C and D)? (3) Does the Nup96sim and Nup160sim double introgression lead to lethality when both are homozygous (or hemizygous) in the D. melanogaster background (Figure 1E)? Based on these three tests, we ask whether the double introgression of Nup96sim and Nup160sim is necessary and sufficient condition for the incompatibility to the gene(s) on the D. melanogastere X chromosome. Dominance of the genes and the possible involvement of different genes to the hybrid lethality will also be discussed.

Figure 1 Genotypes examined previously and in this study. Pairs of bars represent chromosomes X, 2, 3, and 4 (left to right). Open bars (dashed if the presence is not obligate) indicate chromosomes/regions from D. melanogaster, and gray bars indicate chromosomes/regions from D. simulans. D. simulans alleles of Nup160 and Nup96 and the deficiencies on D. melanogaster chromosomes are also indicated. (A) Flies of this genotype all die according to Tang and Presgraves (2009) and Sawamura et al. (2010). (B) Flies of this genotype all die according to Presgraves et al. (2003). (C, D) These flies are viable according to the present analysis. (E) Flies of this genotype all die according to the present analysis. The genotypes in (A) and (B) are usually males carrying one X chromosome from D. melanogaster, but females carrying two D. melanogaster X chromosomes can also be obtained using the attached-X system (Presgraves et al., 2003; Tang & Presgraves 2009). The genotypes in (C), (D), and (E) are females carrying two D. melanogaster X chromosomes or males carrying one D. melanogaster X chromosome.

Figure 2 Construction of chromosome P[w+ Nup96sim] (68A4) e Nup98-96339. A genomic fragment of ~20.9 kb, including three open reading frames (CG10208, Nup98-96, and mbc), was amplified from DSM1-010P23, a D. simulans bacterial artificial chromosome clone established by the National BioResource Project Drosophila (Murakami et al. 2008), by polymerase chain reaction using the primers LA-Ascl-F (5'-AG-GCGGGGCTTACTTGCCGGAACACTGCACTCAG-3'), LA-BamHI-R (5'-CGCGGATCCAGGACACCTCACTGAGTGATTG-3'), RA-BamHI-F (5'-CGCGGATCCAGGACACCTCACTGAGTGATTG-3'), RA-Pad-R (5'-ACCTTAATACTACGACCCGGCATAGTCTGTC-3'). This fragment was subcloned into the vector attB-P[acman]-CmR by homologous recombination (Venken et al. 2006). The construct was injected into embryos of D. melanogaster strain y sc v P[y+T7.7 = nos-phiC31/int. NSL/X; P[y+T7.7 = CaryPlattP2 to allow for phiC31-targeted, site-specific recombination into the attP landing site (cytological position 68A4 on chromosome 3) (Groth et al. 2004; Bateman et al. 2006; Bischof et al. 2007). The resultant transgene is abbreviated as P[w+ Nup96sim] in the present report.

A P[w+ Nup96sim] e Nup98-96339 chromosome was made by recombination between P[w+ Nup96sim] and e Nup98-96339 chromosomes in the w genetic background (Figure 2). Here w+ (68A4; red eye color) and e (93C7-D1; ebony/dark body color) were used as visible markers, and Nup98-96 is at 95B1-5. To confirm that the recombinant chromosome carried the Nup98-96339 mutation and that it was not lost by rare double recombination between e and Nup98-96339, P[w+ Nup96sim] was removed from the established chromosome by further recombination with a wild-type chromosome using the w+ and e markers. The resultant chromosome again exhibited recessive lethality that was not complemented by the Nup98-96 deficiencies (Df(3R)Exel9014 and Df(3R)BSC489), thus confirming that the chromosome examined carried Nup98-96339. A balancer chromosome, TM3, was used to isolate the recombinant chromosome in a heterozygous state, and CyO and SM1 were used as a chromosome 2 balancer. Int(2L)D+s is a chromosome 2 D. simulans introgression covering two cytological regions that include Nup160sim (Sawamura et al. 2000). Of note, the Int(2L)D+s introgression also carries other Nup loci (Nup107 and Nup154), but we do not believe that this could affect our overall conclusion of this study. When

MATERIALS AND METHODS

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necessary, only $Nup160^{imm}$ was made hemizygous by a deficiency of the $Nup160$ locus, $Df(2L)Nup160M190$ (Maehara et al. 2012).

**RESULTS**
First, we established a $D. melanogaster$ line carrying an extra segment of $D. simulans$ chromosome 3 (including $CG10208$, $Nup98$-96, and $mbc$) inserted at cytological position 68A4 of the same chromosome. Note that $Nup98$-96 is a dicistronic gene that produces the proteins $Nup98$ and $Nup96$ by autoproteolysis (Presgraves et al. 2003). Then, the endogenous $Nup98$-96 at 95B1-5 of the line was replaced by the recessive lethal $Nup98-96^{139}$ mutant allele (Figure 2), which has a stop codon at amino acid position 1726 (therefore, only $Nup96$ was affected; Presgraves et al. 2003). Thus, we obtained a $D. melanogaster$ chromosome 3 carrying $Nup96^{sim}$ instead of the $D. melanogaster$ wild-type allele of $Nup96$. The resultant chromosome ($P(w^{+} Nup96^{sim}) e Nup98-96^{139}$) is referred to as the $Nup96^{sim}$ introgression. Both male and female $Nup96^{sim}$ introgression homozygotes (and hemizygotes) were viable and fertile, and the strain homozygous for $Nup96^{sim}$ could be maintained indefinitely. Although females that were homozygous for $Nup96^{sim}$ and hemizygous over $Df(3R)BSC489$ exhibited lower fertility than heterozygous controls ($x^2 = 94.5, P < 0.001$ and $x^2 = 6.6576, P < 0.05$, respectively), fertility was not decreased in $Nup96^{sim}$ hemizygotes over $Df(3R)Exel9014$ ($x^2 = 1.5958, P > 0.2$) (Table 1). Therefore, $Nup96^{sim}$ does not lead to female sterility in the $D. melanogaster$ genetic background. We note the possibility that the chromosome harboring $Nup96^{sim}$ might have a second-site recessive gene or genes responsible for lower female fertility.

Next, to examine possible synergistic and/or additive effects of $Nup160^{imm}$ and $Nup96^{sim}$ introgression, we produced $w; Int(2L)D+S$, $Nup160^{imm}/CyO; Nup96^{sim}/+$ males by conventional crosses. Then, these males were crossed to females heterozygous for a balancer and a mutation (or a deficiency) of $Nup160$ or $Nup98$-96. If the introgressions were behaving similar to the F1 hybrid, then $Nup160^{imm}/(Nup160^{imm} or Df-Nup160)$; $Nup96^{sim}/+$ is expected to be lethal; however, that is not what is observed. Instead, the $Nup160^{imm}$ homozygotes (or hemizygotes) were viable in the $Nup96^{sim}$ heterozygous background (Figure 1C and Table 2). If the introgressions were behaving similar to the F1 hybrid, then $Nup160^{imm}/+/Nup96^{sim}/(Nup96^{sim} or Df-Nup96)$ is expected to be lethal; however, that is not what is observed. Instead, the $Nup96^{sim}$ homozygotes (or hemizygotes) were viable in the $Nup160^{imm}$ heterozygous background (Figure 1D and Table 3). Thus, the $Nup96^{sim}$ and $Nup160^{imm}$ double introgression did not lead to lethality when one was heterozygous and the other homozygous (or hemizygous).

Finally, we attempted to make a strain carrying both $Nup160^{imm}$ and $Nup96^{sim}$ introgressions maintained with chromosome 2 and 3 balancers but were not successful, presumably because $Int(2L)D+S$ can cause dominant male semisterility in some genetic backgrounds (S. Parhad, personal communication). Therefore, we could not test the viability/fertility of $Nup96^{sim}$ and $Nup160^{imm}$ double introgression homozygotes. Instead, we made $w; Int(2L)Nup160M190/SIM1; Nup96^{sim}/TM3$ females and $w; Int(2L)D+S$, $Nup160^{imm}/SIM1; Nup96^{sim}/+$ males by conventional crosses and crossed them. $Int(2L)D+S$, $Nup160^{imm}/ Df(2L)Nup160M190$; $Nup96^{sim}/+$ flies were viable as we previously noted (Table 2), although hemizygosity of $Nup160^{imm}$ might have reduced their viability (Table 4). Unexpectedly, we found that $Int(2L)D+S$, $Nup160^{imm}/ Df(2L)Nup160M190$; $Nup96^{sim}/+$ male parental genotypes for the $Nup96^{sim}$ introgression were fertile (Table 1), in contrast to what has been observed for the $Nup160^{imm}$ introgression, for which eggs produced by homozygotes (or hemizygotes) display karyogamy

### Table 1: Hatchability of eggs from females crossed with wild-type $D. melanogaster$ males

| Maternal Genotype | Number of Eggs | Hatchability, % |
|-------------------|---------------|-----------------|
| $Nup96^{sim}$ heterozygotes over TM3 | 200 | 95.5 |
| $Nup96^{sim}$ homozygotes | 200 | 53.0 |
| $Nup96^{sim}$ hemizygotes over $Df(3R)Exel9014$ | 200 | 92.5 |
| $Nup96^{sim}$ hemizygotes over $Df(3R)BSC489$ | 200 | 88.5 |

* The full genotype of $Nup96^{sim}$ is $P(w^{+} Nup96^{sim}) e Nup98-96^{339}$.

### Table 2: Viability of flies homozygous (or hemizygous) for $Nup160^{imm}$ and heterozygous for $Nup96^{sim}$

| Maternal genotype | Number of Flies |
|-------------------|-----------------|
| $w; Int(2L)D+S$, $Nup160^{imm}/CyO$ Genotype | |
| $Nup160^{imm}/+$; $+/+$ | |
| Females | 132 | 202 |
| Males | 146 | 206 |
| $w; Df(2L)Nup160M190/CyO$ Genotype | |
| $(Nup160^{imm}$ or $Df$-$Nup160$)/+$; $+/+$ | |
| Females | 180 | 201 |
| Males | 155 | 188 |
| Segregation ratio expected | 2 | 2 | 1 | 1 |

* Crossed with $w; Int(2L)D+S$, $Nup160^{imm}/CyO$; $Nup96^{sim}/+$ males. The balancer $CyO$ has $Cy$ as a dominant marker.

* Calculated as (number of flies in the fourth class) divided by (number of flies in the third class).

* The viability of $Int(2L)D+S$ homozygotes was low because of linked recessive lethals that presumably accumulated on the chromosome.
**Table 3** Viability of flies heterozygous for Nup160<sup>sim</sup> and hemizygous for Nup96<sup>sim</sup>

| Maternal genotype<sup>b</sup> | Number of Flies | Number of Flies<sup>b</sup> |
|-----------------------------|-----------------|-----------------------------|
|                             | Cy w Sb | Cy w<sup>+</sup> Sb<sup>+</sup> | Cy w<sup>+</sup> Sb<sup>+</sup> | Cy w<sup>+</sup> Sb<sup>+</sup> | Cy<sup>+</sup> w Sb | Cy<sup>+</sup> w<sup>+</sup> Sb | Cy<sup>+</sup> w<sup>+</sup> Sb<sup>+</sup> (Viability<sup>b</sup>) |
| w; Nup8-96<sup>339</sup>/TM3 |      |                       |                |                       |               |                       |                                  |
| +/++; +/+/l(3)Nup96/               | 38     | 50                       | 84             | 72                     | 102           | 84                     | 72 (0.86)                      |
| Females                       |        |                          |                |                       |               |                       |                                  |
| w, Df(3R)85C489/TM6C         | 61     | 70                       | 62             | 87                     | 109           | 90                     | 69 (1.11)                      |
| Genotype                     |        |                          |                |                       |               |                       |                                  |
|                               |        |                          |                |                       |               |                       |                                  |
| +/+; +/+/Df-Nup96/             | 123    | 63                       | 142            | 98                     | 151           | 62                     | 92 (0.65)                      |
| Females                       |        |                          |                |                       |               |                       |                                  |
| +/+; +/+/Df-Nup160/; Df-Nup96/  | 117    | 28                       | 106            | 85                     | 128           | 76                     | 65 (0.61)                      |
| Males                         |        |                          |                |                       |               |                       |                                  |
| Segregation ratio expected    | 1      | 1                        | 1              | 1                      | 1             | 1                      | 1                                |

<sup>a</sup> Calculated as (number of flies in the eighth class) divided by (number of flies in the fourth class).

<sup>b</sup> They were crossed to w; Int(2L)D; Nup160<sup>sim</sup>/CyO; Nup96<sup>sim</sup>/ males. The balancers TM3 and TM6C have Sb (and Ser in the former) as a dominant marker. l(3)Nup96 stands for a recessive mutation of the Nup96 gene, Nup98-96<sup>339</sup>.

**Table 4** Viability of flies hemizygous for Nup160<sup>sim</sup> and homozygous for Nup96<sup>sim</sup>

| Maternal genotype<sup>c</sup> | Number of Flies<sup>a</sup> | Number of Flies<sup>b</sup> |
|-------------------------------|-----------------------------|-----------------------------|
|                               | Cy w Sb | Cy w<sup>+</sup> Sb<sup>+</sup> | Cy w<sup>+</sup> Sb<sup>+</sup> | Cy w<sup>+</sup> Sb<sup>+</sup> | Cy<sup>+</sup> w Sb | Cy<sup>+</sup> w<sup>+</sup> Sb | Cy<sup>+</sup> w<sup>+</sup> Sb<sup>+</sup> (Viability<sup>b</sup>) | Cy<sup>+</sup> w<sup>+</sup> Sb<sup>+</sup> (Viability<sup>b</sup>) |
| w; Df(2L)Nup160M190/SM1; Nup96<sup>sim</sup>/TM3 |      |                       |                |                       |               |                       |                                  |                                  |
| (Nup160<sup>sim</sup> or Df-Nup160<sup>sim</sup>/+); Nup96<sup>sim</sup>/+; Nup96<sup>sim</sup>/+ (TM3) | 436<sup>d</sup> | 533                       | 423             | 163                    | 137           | 98<sup>e</sup>                     | 1 (0.01)                        | 0 (0)                        |
| Females |        |                          |                |                       |               |                       |                                  |                                  |
| (Nup160<sup>sim</sup> or Df-Nup160<sup>sim</sup>/+); Nup96<sup>sim</sup>/+; Nup96<sup>sim</sup>/+ (TM3) | 442<sup>d</sup> | 547                       | 452             | 190                    | 145           | 177                     | 8 (0.05)                        | 0 (0)                        |
| Males   |        |                          |                |                       |               |                       |                                  |                                  |

<sup>a</sup> w<sup>+</sup> means flies carrying two w<sup>+</sup> markers; distinguished by their darker eye color. A few flies ambiguous for the Cy phenotype were excluded.

<sup>b</sup> Calculated as (number of flies in the seventh or eighth class) divided by (number of flies in the sixth class).

<sup>c</sup> Crossed with w; Int(2L)D; Nup160<sup>sim</sup>/SM1; Nup96<sup>sim</sup>/+ males. The balancers SM1 and TM3 have Cy and Sb (and Ser) as dominant markers, respectively.

<sup>d</sup> One was ebony presumably caused by a rare male recombination or a spontaneous mutation.

<sup>e</sup> One was a gynandromorph.
failure and female pronuclei never fuse to wild-type male pronuclei (Sawamura et al. 2004). Although Nup96 and Nup160 are functionally and structurally in close proximity in the Y-shaped Nup107–160 complex, the effects of interspecific substitution of these two components differed. The structural position of Nup96 and Nup160 might reflect the functional difference; Nup160 is on the surface of the pore ring (Bilokapic and Schwartz 2012; Szymborska et al. 2013) and might have more interactions with other proteins important for NPC function.

We found that flies with genotypes indicated in Figure 1, C and D were viable (Table 2 and Table 3), in contrast to the lethality observed for those with genotypes indicated in Figure 1, A and B (Presgraves et al. 2003; Tang and Presgraves 2009; Sawamura et al. 2010). The primary difference between these flies is the genetic background, with the remaining autosomal genes being from D. melanogaster in our flies and from D. melanogaster and D. simulans (hypozygous) in the previous studies. Apparently the presence of additional autosomal D. simulans genes is necessary to cause lethality, and these genes are dominant to the D. melanogaster alleles. Thus, more genes (maybe encoding other Nups) are involved in this hybrid incompatibility. Nup107 and Nup154 are excluded from the candidates because Int (2L)D+S also carries these genes from D. simulans but did not exhibit the dominant effect. One candidate for the interactor is Nup75, presumably the Drosophila homolog of Nup85. Further investigation of this system is necessary to better understand the genetic mechanisms of reproductive isolation.

Interestingly, dominance of Nup96sim was changed by the presence of a balancer TM3 (Table 4). Reproductive isolation might be easily affected by the genetic background, as has been suggested in the other hybrid incompatibility (Uhr vs. Hmr) in the same species cross (Matute et al. 2014; Shirata et al. 2014). Finally, double introgression carrying homozygous Nup96sim and hemizygous Nup160sim resulted in lethality in the hybrids (Table 4 and Figure 1E). This is the first evidence suggesting that Nup96sim and Nup160sim are two components of the same incompatibility.

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