Knockout Targeting of the Drosophila *Nap1* Gene and Examination of DNA Repair Tracts in the Recombination Products

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ABSTRACT

We used ends-in gene targeting to generate knockout mutations of the nucleosome assembly protein 1 (*Nap1*) gene in *Drosophila melanogaster*. Three independent targeted null-knockout mutations were produced. No wild-type NAP1 protein could be detected in protein extracts. Homozygous $Nap1^{KO}$ knockout flies were either embryonic lethal or poorly viable adult escapers. Three additional targeted recombination products were viable. To gain insight into the underlying molecular processes we examined conversion tracts in the recombination products. In nearly all cases the *I-Sce*I endonuclease site of the donor vector was replaced by the wild-type *Nap1* sequence. This indicated exonuclease processing at the site of the double-strand break (DSB), followed by replicative repair at donor-target junctions. The targeting products are best interpreted either by the classical DSB repair model or by the break-induced recombination (BIR) model. Synthesis-dependent strand annealing (SDSA), which is another important recombinational repair pathway in the germline, does not explain ends-in targeting products. We conclude that this example of gene targeting at the *Nap1* locus provides added support for the efficiency of this method and its usefulness in targeting any arbitrary locus in the Drosophila genome.

THE completion of the genome sequence provides unlimited access to all genes of *Drosophila melanogaster* (Adams *et al.* 2000). Nevertheless, despite nearly a century of Drosophila genetics, there are many Drosophila genes for which corresponding mutants are still unavailable. Means to overcome the drawback had been site-selected transposon mutagenesis (Ballinger and Benzer 1989; Kaiser and Goodwin 1990) and RNA-mediated interference (RNAi; Kennerdell and Carthew 1998). While transposon mutagenesis involves elaborate PCR screening, RNAi generates only gene-specific phenocopies of loss-of-function mutations and does not always cause a true null phenotype. Therefore, methods of gene knockout targeting have been developed. Drosophila gene targeting is accomplished by two alternative techniques (Gloor *et al.* 1991; Rong and Golic 2000). Both take advantage of the fly's endogenous homologous recombination machinery in the germline. One method utilizes a *P*-element-induced double-strand break in a target gene, which then is repaired from an ectopic donor construct by means of synthesis-dependent strand annealing (SDSA; Nassif *et al.* 1994). *P*-induced gap repair was developed by Engels and colleagues (Gloor *et al.* 1991; for a review see Lankenau 1995; Lankenau and Gloor 1998). The drawback of *P*-induced gap repair is the need for a suitable *P* element tightly linked to the gene to be modified. Unfortunately, not all (*i.e.*, 20%) of the Drosophila genes are available as *P* insertions (Spradling *et al.* 1999). The other technique resembles knockout targeting in mouse embryonic stem cells (Capecchi 1989a; Rong and Golic 2000). Rong and Golic's approach now can target a mutation to any arbitrary locus in the Drosophila genome (Rong *et al.* 2002). The method involves four components: (1) a transgene that expresses a heat-shock-inducible site-specific recombinase (FLP); (2) a second transgene that expresses a heat-shock-inducible site-specific endonuclease (*I-Sce*I); (3) a transgenic donor vector that contains recognition sites for both enzymes in addition to the *white* gene as a positive selection marker; and (4) the native wild-type target gene. Through heat shock, the FLP recombinase excises a circular episome containing the *white* marker gene and an *in vitro* modified donor gene. The extrachromosomal DNA molecule is linearized within the modified donor gene through the activity of the heat-induced *I-Sce*I endonuclease.

Rong and Golic (2000) pioneered the new approach first at the *yellow* gene. Targeting additional genes at central chromosomal positions demonstrated that arbitrary loci can be modified (Rong and Golic 2001; Rong *et al.* 2002; Seum *et al.* 2002). As a further example of gene targeting in Drosophila, we set out to target an essential gene relevant for our future research. Because of its chromosomal location (1.4 Mb to the telomere),

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the expectation of obtaining a visible phenotype, and the lack of null mutations, we chose to target the nucleosome assembly protein 1 (*Nap1*) gene (Ito *et al.* 1996). Drosophila NAP1 has been shown to be required for chromatin assembly *in vitro* and was found to be associated with core histones H2A and H2B as a chaperone in embryonic extracts (Ito *et al.* 1996). The only *in vivo* data were obtained from the yeast *Nap1* homolog, but these indicated a role in cell cycle regulation rather than in chromatin assembly (Kellogg and Murray 1995). The generation of a *Nap1* knockout mutant would also be a first step toward determining its *in vivo* function in Drosophila. Six targeted knockout mutations ($Nap1^{KO}$) with three complete *Nap1* gene knockouts were obtained. Three homozygous knockout mutations expressed no NAP1 protein and showed a semilethal phenotype.

Three additional targeted $Nap1^{KO}$ recombinants were viable. To understand the molecular differences between the six targeted $Nap1^{KO}$ mutants we performed a detailed molecular analysis of these recombination products. We constructed the targeting vector such that the *Nap1* donor gene included protein-function-destroying mutations, which simultaneously introduced five molecular repair-tract markers. Because ends-in targeting produces target-gene duplications, each *Nap1* copy and the five corresponding tract markers were duplicated at each targeted event. Thus from the six targeting events we monitored 60 marker positions. The 18-bp *I-Sce*I endonuclease site where the *Nap1* donor was cleaved for double-strand break (DSB)-induced targeting was in all but one case replaced by wild-type *Nap1* sequence. This indicated that exonuclease processing at the DSB and replicative repair at the donor-target junctions had taken place. The results are discussed mechanistically with relation to major models of recombinational DNA repair. Either the classical DSB repair model involving double Holliday junctions (Szostak *et al.* 1983) or the break-induced recombination (BIR) model (Malkova *et al.* 1996) best explains the tract data. We conclude that this example of targeted gene modification at the *Nap1* locus was efficient and confirmed the expectation that any arbitrary locus can be targeted. The described procedure is powerful and it clearly represents a general method for targeted mutagenesis.

MATERIALS AND METHODS

Drosophila: Genetic symbols are defined in standard reference works (Lindsley and Zimm 1992; FlyBase 1999). Genomic DNA sequences of the *Nap1* gene and flanking sequences were accessed via http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/7227.html and derived from the assembled and annotated genome sequence of *D. melanogaster* as available in GenBank (Myers *et al.* 2000). Fly stocks used for the targeting screen were a gift from Kent Golic (Rong and Golic 2001; Rong *et al.* 2002):

1. *y w; P{ry*$^{+}$*, 70 FLP}4 P{v*$^{+}$*, 70 I-SceI}2B Sco/S*^{2} *CyO*
2. w^{1118}*; P{ry*$^{+}$ *70 FLP}10;* + (strong constitutive expression of FLP).

All experimental fly stocks were tested for absence of endosymbiotic, cytoplasmically inherited Wolbachia bacteria using published *Wolbachia pipientis*-specific 16S RNA PCR primers (O'Neill *et al.* 1992).

Construction of donor plasmid and microinjection: On the basis of the genome DNA sequence of Drosophila, recombinant PCR (Higuchi 1990) was used to generate a 4.275-kb *Nap1*-containing fragment from genomic DNA of Canton-S wild-type flies. The following oligonucleotides were used:

1. (*Acc65*I) 5′ CGCGGTACCaagcagcaaaggcaacgcaaaatgac 3′
2. (*Not*I) 5′ CGCGCGGCCGCacgcataaaattactgattccgcgctaag 3′
3. (*I-Sce*I) 5′ TAGGGATAACAGGGTAATccttgccctcgatgatctcc 3′
4. (*I-Sce*I) 5′ ATTACCCTGTTATCCCTAtggacccgccgaggaga ag 3′
5. (*Hin*dIII) 5′ GTTGCAGGACTCGGGGTCAACGTGaagCT TCGGCTGGGGCGTCCATTG 3′
6. (*Hin*dIII) 5′ CAATGGACGCCCCAGCCGAAGcttCACGTT GACCCCGAGTCCTGCAAC 3′
7. (*Bcl*I) 5′ GCTTCTTGCGGATGGTCTgaTCACAGTAAGG TTCATC 3′
8. (*Bcl*I) 5′ GATGAACCTTACTGTGAtcAGACCATCCGCAA GAAGC 3′.

The isolated DNA fragment finally encompassed five molecular markers (*Xho*I−, *Hin*dIII+, *I-Sce*I+, *Bcl*I+, and *Sal*I−), which destroyed the open reading frame (ORF) structure to the left and right of the *I-Sce*I site such that a *Nap1*-targeted knockout duplicate could be not functional. The mutated *Nap1* fragment was cloned into the *Acc65*I/*Not*I sites of the pTV2 vector obtained from Kent Golic (Rong *et al.* 2002; Figure 1A). The pTV2-$Nap1^{mut}$ DNA was microinjected into w^{67c23}; *TM6 Ubx/ Sb P{ry*$^{+}$ *Δ2-3}99B* embryos. An insertion of pTV2-$Nap1^{mut}$ into the *TM6 Ubx* balancer chromosome was further used in a screen for *Nap1* knockout mutations (Figure 1B).

Analysis of recombinant flies: *Polytene in situ hybridization:* The w^{hs} gene from the pTV2-vector (Rong *et al.* 2002) was biotinylated by random priming and used as a probe for *in situ* hybridization to polytene chromosomes as described (Lim 1993; Figure 2A).

Southern blot: Southern blots were performed according to standard protocols (alkaline transfer onto positively charged nylon membranes; Ausubel *et al.* 1995) and hybridized to a biotinylated *Nap1* probe (Figure 2C). Detection using streptavidin-alkaline phophatase and CDP star as substrate was performed using the detector system (KPL, Gaithersburg, MD).

PCR analysis: Genomic DNA of heterozygous flies was used to track the molecular markers by performing PCR with three different primer combinations and subsequent restriction digests (Figure 4, A and B). Primer pI primes specifically to the 5′ genomic region upstream of the distal *Nap1* duplicate; the PCR product of primers pI and pII is therefore used to follow the markers on the distal (*i.e.*, telomeric) side. Primer pIV is specific for the 3′ genomic region downstream of the proximal *Nap1* duplicate, so that the PCR product of pIII and pIV serves to characterize the proximal *Nap1* duplicate. Primers II and III hybridize to all three different *Nap1* copies (distal, proximal, and wild type). This PCR product was analyzed by a subsequent double digest with *Hin*dIII and *Bcl*I to prove the presence of the introduced restriction sites and to show the absence of the donor construct at the same time. Primers are as follows:

pI: CTCGAATTCTAGCACCCATGATACCATCTTATGG;
pII: CGCTCTAGAAATCCAGCCACATCAACCTACTGA;
pIII: CGCGCGGCCGCACGCATAAAATTACTGATTCCGCG CTAAG;
pIV: CGCTCTAGAATTGATGGAACGCACTCGAAACTG.

Sequencing: Distal- and proximal-specific PCR fragments (see *PCR analysis*) were gel purified and PCR fragments spanning the region of the *Nap1* construct containing the *I-Sce*I site were generated using primers pV and pVI (pV, CTCGAATTCA CTATTGGCCAGCAAACTCA; pVI, CTCTCTAGACTACAGC TGCAGCACCTGAATATCGA). The PCR fragments were directly sequenced using an ALF sequencer.

Western analysis: Protein extracts were prepared from Drosophila Oregon-R wild-type females and from homozygous mutant *Nap1* females or dissected ovaries. Equal amounts of protein were separated on a 15% polyacrylamide gel and blotted using standard procedures. As primary antibody we used anti-NAP1 (Li *et al.* 1999) and anti-Rp40 as a loading control (Torok *et al.* 1999). As secondary antibody we used anti-rabbit IgG coupled to HRP (Dianova). Secondary antibody was visualized by enhanced chemiluminescence (Perkin-Elmer, Norwalk, CT) and exposure to X-ray films (Figure 3, A and B).

Immunostaining of ovaries and confocal laser scanning microscopy: Ovaries were dissected from wild-type (Oregon-R) and homozygous mutant *Nap1* females and the tissue was fixed for 40 min in 4% formaldehyde, 0.5% Tween 20, and 1× PBS followed by three washes in 1× PBS. After blocking in 1× PBS, 1% BSA, 0.1% Tween 20, ovaries were incubated with anti-NAP1 antibody. After three washes with blocking solution, incubation with the secondary antibody (anti-rabbit Cy5; Dianova) followed in combination with 10 μg/μl propidium iodide and 100 μg/μl RNase A to visualize DNA and phalloidin FITC to visualize F-actin. Finally, ovaries were washed in blocking solution, in 1× PBS, 1% BSA, and in Slow Fade Light Component C (Molecular Probes, Eugene, OR). Preparations were mounted in Slow Fade Light Component A and analyzed with a Zeiss LSM410 confocal microscope (Figure 3, C and D).

RESULTS

Design of the *Nap1* knockout construct and the genetic screen: We used the *D. melanogaster Nap1* gene mRNA sequence (GenBank accession no. U39553; Ito *et al.* 1996) to identify genomic DNA sequences flanking this gene. We identified a 4.5-kb fragment within a Drosophila scaffold section of the complete genomic sequence (accession no. AE003462). The intron/exon structure of *Nap1* and its location within the 4.5-kb genomic fragment was roughly confirmed with the GENSCAN software. On the basis of this sequence we designed eight oligonucleotide primers, which were simultaneously used for three purposes:

1. The isolation of a 4.3-kb PCR fragment from genomic DNA of wild-type Drosophila flies containing the *Nap1* gene in a central position.
2. The introduction of mutations into the wild-type *Nap1*-coding region, which destroys the function of its protein product. The mutations flank an introduced *I-Sce*I endonuclease cutting site on both sides (Figure 1A).
3. The mutations further introduced *Hin*dIII and *Bcl*I as artificial restriction endonuclease cutting sites and a *Xho*I and a *Sal*I site were destroyed. These sites were used to track the DNA repair activities responsible for targeted gene knockout events *in vivo* (Figure 1A).

With these eight primers we isolated and simultaneously mutagenized a 4.3-kb *Nap1* fragment from genomic DNA by recombinant PCR (Higuchi 1990). The fragment was introduced into the pTV2 *P*-element vector (Figure 1A; Rong *et al.* 2002), and transgenic flies were established containing the *Nap1*/pTV2 donor construct integrated in a third balancer chromosome (Tm6, *Ubx*). These flies were used to screen for knockout events as shown in Figure 1B. Figure 1C shows the expected result of a knockout ("knock-in") event at the *Nap1* locus. The absence or presence of the *I-Sce*I cutting site was not foreseeable. However, on the basis of published conversion frequency studies (Gloor *et al.* 1991; Preston and Engels 1996) we expected exonucleolytic processing of the *I-Sce*I-induced DSB to result in wild-type sequence at the donor/target junctions.

We carried out three targeting screens as shown in Figure 1B, each using slightly different heat-shock conditions. Table 1 shows the results of the screens. With the red eye-color phenotype as a positive marker we identified eight recombination events with six targeted events and three independent null-mutation *Nap1* knockout products.

Verification of targeted knockout events: Two of the eight recombination events genetically segregated with the X chromosome and were not further analyzed. The six remaining recombination events segregated with the second chromosome where the *Nap1* gene is located. None of them expressed a mosaic red/white eye-color phenotype when combined with a constitutively expressed FLP recombinase source (Rong and Golic 2001). Nonmosaicism confirmed that they were good candidates for targeted recombination events. Using the w^{hs} gene as a probe for *in situ* hybridization to polytene chromosomes, we located the w^{hs} gene at the *Nap1* locus on the second chromosome (Figure 2A). Southern blot analysis confirmed the expected knockout duplications (Figure 2, B and C). Genomic DNA of heterozygous $Nap1^{+}/Nap1^{KO}$ flies was digested either with *Bcl*I, diagnostic for the distal part (Figure 2B, bottom), or with *Hin*dIII, diagnostic for the proximal part (Figure 2B, top) of the predicted knockout duplication. In addition to the 12.9-kb *Bcl*I fragment diagnostic for the *Nap1* wild-type gene, the recombinant flies $Nap1^{KO1}$, $Nap1^{KO2}$, $Nap1^{KO5}$, and $Nap1^{KO6}$ showed two bands (2.3 and 8.5) derived from the targeted *Nap1* locus, identifying the incorporated *Bcl*I site. Next to the wild-type fragment (8.3 kb, Figure 2, B and C) the *Hin*dIII site of the targeted *Nap1* duplication was found in $Nap1^{KO1}$–$Nap1^{KO4}$ but not in $Nap1^{KO5}$ and $Nap1^{KO6}$ (11.2- and 4.5-kb *vs.* 16.7-kb fragments, Figure 2, B and C). The DNA blot results indicated that the $Nap1^{KO1}$ and $Nap1^{KO2}$ alleles represented the anticipated knockout products. Because $Nap1^{KO3}$–$Nap1^{KO6}$ did not reveal identical fragment patterns in the DNA blot analysis, the recombination tracts of the six knockout events were studied to gain insight into the underlying DNA repair pathway (see below).

Absence of NAP1 56-kD protein in knockout mutants leads to lethality: We performed Western blot analyses

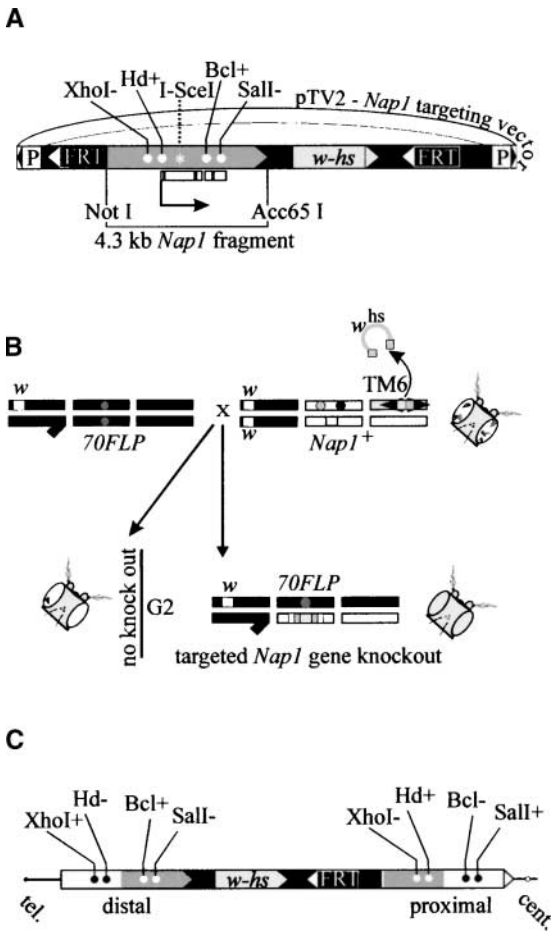

FIGURE 1.—Strategy for *Nap1* knockout targeting. (A) Structure of the donor targeting vector. The pTV2 plasmid contains a nonautonomous *P*-transposable element. The *P* vector carries the *white-hs* (*w-hs*) marker gene, two FRT sites in direct orientation for FLP-mediated episomal excision, and the *in vitro* mutagenized *Nap1* gene. The 4.3-kb genomic fragment containing the *Nap1* gene was inserted into pTV2 at *Not*I and at *Acc65*I. The intron-exon structure is as indicated. *Nap1* was mutagenized by recombinant PCR: The center of *Nap1* contains an *I-Sce*I site. On the left side of the *I-Sce*I site, the inserted *Hin*dIII site (Hd+) introduces a reading frameshift into the open reading frame of *Nap1* at the sixth most N-terminal amino acid. On the right side of the *I-Sce*I site, the inserted *Bcl*I restriction site introduces a reading frameshift that truncates the NAP1 protein and destroys the nuclear localization signal. Two other restriction enzyme markers, *Xho*I and *Sal*I, were deleted from the donor construct to support recombination tract analysis (*Xho*I$^-$, *Sal*I$^-$). The intron/exon structure and its transcriptional orientation of *Nap1* are indicated. (B) Cross to generate a targeted gene knockout of *Nap1*. The TM6, *Ubx* balancer chromosome (TM6) in a G_1 female fly contains the transgenic *Nap1 P*-element donor construct. The wild-type *Nap1* gene (*Nap1*$^+$) is located on both second chromosome homologs (only one copy is shown, as a rectangle). One of these homologs contains two heat-shock-inducible transgenes: FLP recombinase (shaded circle) and *I-Sce*I endonuclease (solid circle). The X chromosomes are homozygous for a *white* (*w*) mutation. Upon heat shock during larval development, FLP and *I-Sce*I produce the extrachromosomal targeting molecule. The female fly is crossed to a transgenic male with strong constitutive expression of FLP recombinase (*70FLP*, dark-shaded circle). Because of the efficiency of constitutive FLP expression, offspring flies that do not carry a targeted insert but still contain an unexcised donor on the TM6, *Ubx* balancer are white eyed with rare colored spots. (C) Expected *Nap1* knockout targeting duplication (knock-in). *Nap1* knockout sequences (shaded rectangle) are marked by *Bcl*I (Bcl+) and *Hin*dIII (Hd+). Only one FRT site remains, stabilizing the *w-hs* gene under constitutive FLP expression. tel., telomere; cent., centromere.

to further confirm successful targeting and to show if *Nap1* knockout flies expressed NAP1 protein (Figure 3, A and B). The proximal construct of the targeted *Nap1* duplication was designed by introduction of a *Hin*dIII site such that the ORF of the *Nap1* knockout product would be destroyed close to the initial methionine, thus resulting in no protein product (Figure 1, A and C). The distal part of the knockout duplication introduced a *Bcl*I site to destroy the ORF in the middle of the *Nap1* gene and to damage its nuclear localization signal (Figure 1, A and C). By Western blot analysis, we found that no 56-kD wild-type NAP1 protein can be detected either in whole fly protein extracts or in ovaries of homozygous *Nap1*^{KO1} and *Nap1*^{KO2} knockout flies (Figure 3, A and B). *Nap1*^{KO3} and *Nap1*^{KO4} were not further analyzed because too few homozygous offspring were obtained, which is possibly due to second-site mutations elsewhere in the genome. Homozygous *Nap1*^{KO5} and *Nap1*^{KO6}, however, produced wild-type NAP1 protein (Figure 3B). Southern and repair tract analyses (see below) revealed that these alleles as well as *Nap1*^{KO4} were targeted recombination events in which one of the two *Nap1* duplicates remained wild type (Figures 2, B and C, and Figure 4).

The Drosophila ortholog of *Nap1* in humans was identified as a chaperone factor involved in the assembly of nucleosomes (ISHIMI *et al.* 1984; ITO *et al.* 1996). Because nucleosome assembly is essential for eukaryotic cells the observed absence of wild-type 56-kD NAP1 protein in homozygous *Nap1* knockout flies should result in a phenotype. Indeed, only low percentages of adult homozygous *Nap1*$^{KO1-2}$ flies hatched. The viability of the *Nap1*^{KO} mutants was determined in *Nap1*^{KO}/T(2;3) *Cy Roi Tb* fly stocks. The hatch rate of homozygous *Nap1*^{KO1} knockout flies was by a factor of 5 lower than expected (6.3%), indicating a lethality analogous to perinatal lethal phenotypes of mice. No unusual pupal lethality was observed, suggesting that the limited hatch rates were due to larval or embryonic lethality. Low adult hatch rates were also observed for homozygous *Nap1*^{KO2} flies (7%). In the case of *Nap1*^{KO3}, only a single fly hatched, and, similarly *Nap1*^{KO4} did not produce any homozygous adult flies, which is presumably due to epistatically interacting second-site mutations elsewhere in the genome. Consistent with their expression of wild-type NAP1 protein (Figure 3B), *Nap1*^{KO5} and *Nap1*^{KO6} showed the expected percentage of homozygous flies (30%). Together with the Southern analysis this result indicated that one copy

TABLE 1

Targeted gene knockout mutagenesis screens of the *Nap1* gene

Screen	Age[a] (hr)	Heat-shock conditions	No. of single crosses	No. of scored chromosomes	No. of independent targeted recombination events (nontargeted)	No. of independent targeted *Nap1* knockout-null mutants
1	72	60′ 38°	1,100	165,000	0	0
2	72	60′ 38°	300	45,000	1	0
	120	120′ 38°				
3	48	75′ 37°	450	80,300	5 (2)	3
	72	90′ 37°				

Three independent genetic screens with varied heat-shock conditions were performed to generate targeted knockout-null mutants of the *Nap1* gene. Flies carrying the donor construct on the TM6 balancer chromosome, as well as the heat-shock-inducible FLP recombinase and *I-Sce*I endonuclease as transgenes on the second chromosome, produced the extrachromosomal targeting molecule upon heat shock during larval development. Two exposures to heat shock on the second/third day of development proved to be most efficient in generating targeting events (screen 3). Two nontargeted recombination events, which segregated with the X chromosome, were obtained and were not analyzed further. Three of the six generated targeted recombination events (KO1, KO2, and KO3; compare Figures 3 and 4C) were found to be *Nap1* knockout-null mutants.

[a] Crosses were kept at 25°, G_0 parents were discarded after 24 hr, and embryos and larvae were exposed to one or two heat shocks at controlled times after crosses were set up (screens 1–3).

of the targeted $Nap1^{KO5}$ and $Nap1^{KO6}$ duplications contained wild-type function (Figure 2B and Figure 4). We further crossed homozygous $Nap1^{KO1}$ males and females. Among several crosses only a few produced rare $Nap1^{KO1}$ late-hatching offspring. These flies died after 5–8 days—much earlier than wild-type flies—and no further offspring were generated. The lethal phenotypes therefore were similar to the mutant phenotypes of other genes thought to be important in nucleosome remodeling. For example, *imitation switch* (ISWI) homozygotes, where ISWI is the catalytic subunit of the three essential chromatin-remodeling complexes NURF, ACF, and CHRAC, die as late larvae or early pupae (Varga-Weisz and Becker 1998; Deuring *et al.* 2000).

We further performed immunofluorescence microscopy (Figure 3, C and D). NAP1 protein is known to be abundant in the follicle cells of ovaries. Figure 3, C and D, shows a confocal laser-scan analysis of the localization of NAP1 in wild-type and homozygous $Nap1^{KO1}$ mutant ovaries. NAP1 protein levels are specifically concentrated at the basal pole of follicle cells of wild-type ovaries. This accumulation of NAP1 protein was absent in the homozygous $Nap1^{KO1}$ mutant.

Recombination tracts of knockout alleles: Southern blot analysis of targeted genomes indicated differences between the molecular structure of individual *Nap1* loci (Figure 2, B and C). These results encouraged a more detailed investigation of the introduced recombination tract markers. We analyzed conversion tracts that included five markers (X, H, I, B, and S; Figure 4C) in each copy of the *Nap1* gene of the six recombinant knock-in duplications by means of PCR with genomic DNA and subsequent restriction analysis. Figure 4, A and B, shows an example of this study. First we used a systematic set of side-specific PCR primers to amplify the distal and proximal duplication fragments. We identified four tract classes (Figure 4C). Confirming the Southern blot results (Figure 2), the alleles $Nap1^{KO1}$ and $Nap1^{KO2}$ represented the predicted recombination tracts. $Nap1^{KO3}$ was another true knockout allele, but it possessed a nearly complete donor-derived repair tract except that the *I-Sce*I site was absent. $Nap1^{KO4}$ was a targeted recombination event but it contained one complete wild-type *Nap1* copy at its distal duplication while the proximal duplication was identical to the sequence of the *Nap1* donor construct. $Nap1^{KO5}$ and $Nap1^{KO6}$ contained the same predicted recombination tracts as $Nap1^{KO1}$–$Nap1^{KO3}$ on the distal half whereas the proximal replica was completely wild type. Rong and Golic (2000) observed a frequent replacement of the *I-Sce*I cut site sequences at the termini of the donor with the wild-type genomic sequence. Sequencing of all PCR fragments spanning the *I-Sce*I site (Figure 4C, primers pV and pVI) revealed that all our knockout duplications except the proximal fragment of $Nap1^{KO4}$ contained the wild-type *Nap1* sequence replacing the *I-Sce*I cut site. This suggested that exonuclease activity was involved in processing the terminal heterology of the *I-Sce*I cut, *i.e.*, the removal of at least four nucleotides of single-strand (ssDNA) and 5 bp of dsDNA from the *I-Sce*I site on one side of the episomal DSB, and four nucleotides of protruding ssDNA and 9 bp of dsDNA on the other side (Figure 5).

DISCUSSION

The induction of mutations within genes is tightly coupled to our basic understanding of gene function. Precisely defined mutations are therefore a prerequisite to analyze the function of genes and their phenotypic impacts. Unfortunately, although the Drosophila genome has been mutated at very high density, no mutants

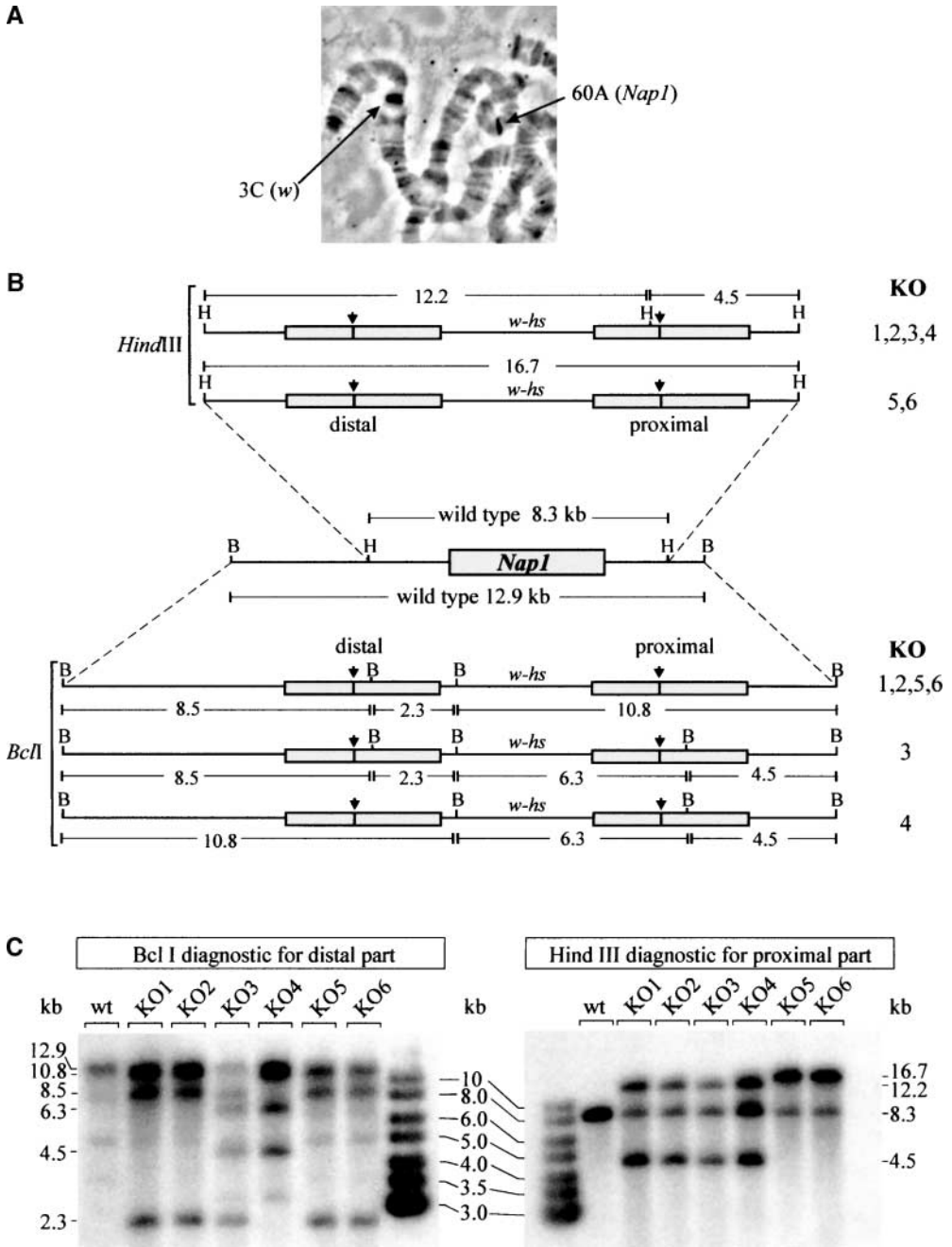

FIGURE 2.—Mapping and verification of targeted recombination events. (A) Localization of w^{hs} at the *Nap1* locus by polytene chromosome *in situ* hybridization. Chromosomes from flies homozygous for the targeted viable $Nap1^{KO5}$ allele were probed with labeled *white-hs* gene DNA. Two signals were detected: one at the 3C locus, which is the endogenous *white* locus, and the other at 60A, which is the *white-hs* insertion at the targeted *Nap1* locus. (B) Map of genomic restriction fragments diagnostic for targeted knockout mutations KO1–KO6. Centered is a sequence-derived *Hin*dIII (H)- and *Bcl*I (B)-based restriction map of the wild-type *Nap1* locus. The upper two restriction maps indicate *Hin*dIII digestion patterns with the 4.5-kb fragment diagnostic for successful targeting of the proximal part of the anticipated targeted knockout gene. The three bottom restriction maps indicate *Bcl*I digestion patterns with the 2.3-kb fragment diagnostic for successful targeting of the distal part of the anticipated targeted *Nap1* gene. Arrows indicate position of the *I-Sce*I cutting site prior to the targeting event. (C) Southern blot analysis. Genomic DNA of wild-type flies and of heterozygous recombinants ($Nap1^{KO1–KO6}$) was digested with either *Bcl*I (left) or *Hin*dIII (right). A wild-type 4.3-kb genomic fragment containing the *Nap1* locus was used as a biotinylated probe. The 2.3- and 8.5-kb *Bcl*I fragments of the recombinant flies $Nap1^{KO1}$, $Nap1^{KO2}$, $Nap1^{KO5}$, and $Nap1^{KO6}$ identify the incorporated *Bcl*I site at the targeted *Nap1* locus. In KO1–KO4 but not in KO5 and KO6, two *Hin*dIII fragments of 12.2 and 4.5 kb prove the introduction of the *Hin*dIII site in the proximal *Nap1* duplicate.

are known for a significant fraction of genes. The *Nap1* gene is one such example where a mutant null allele has not been available. While traditional mutagenesis procedures are based on phenotypic screening (with complex and time-consuming genetic crosses to look for recessive phenotypes), gene targeting requires no prediction of the mutant phenotype. There are convincing arguments that the technique of RNAi (which also does not require knowledge of a phenotype) is simpler on a practical level and therefore is better suited than targeted mutagenesis to overcome the lack of mutants (CARTHEW 2001). However, RNAi generates only gene-specific phenocopies of null mutations but does not always cause a true null phenotype (ADAMS and SEKELSKY 2002). Therefore, altering specific endogenous genes within the metazoan germline represents a foundation for the highest possible level of experimental control over a particular locus of interest. The establishment of mouse embryonic stem (ES) cell lines, techniques transforming vector DNA into ES cells, and advanced methods to produce chimeras and completely ES-cell-derived fetuses trail blazed metazoan targeting and made the mouse a leading model organism (THOMAS and CAPECCHI 1987; CAPECCHI 1989a,b; JOYNER 1995).

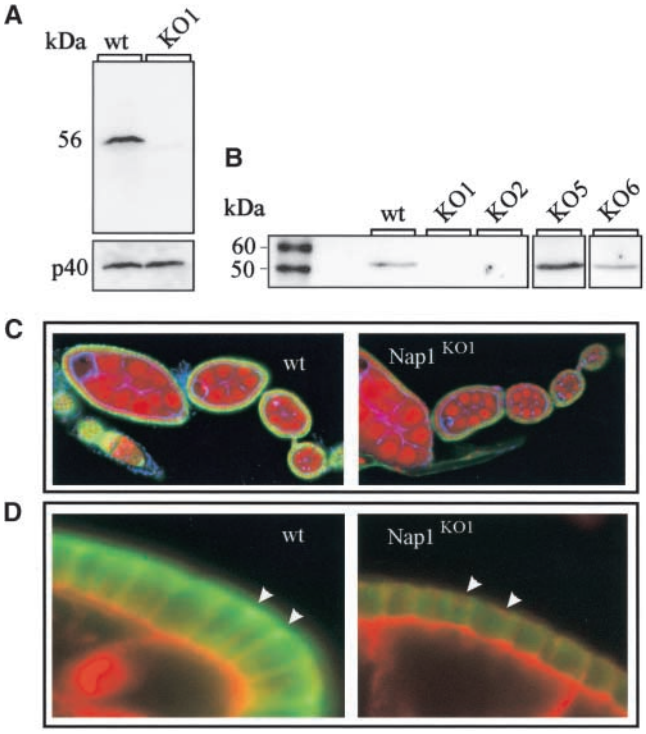

FIGURE 3.—Analysis of NAP1 protein in targeted flies. (A) Western blot analysis of wild-type and homozygous *Nap1*^{KO1} knockout flies. Total protein extracts were obtained from adult female flies and anti-NAP1 antibody was used (LI *et al.* 1999). Anti-p40 antibody served to control for equal loading (bottom; TOROK *et al.* 1999). (B) Western blot analysis of ovaries from wild-type and homozygous *Nap1*^{KO} mutant flies. The genotypes were confirmed by PCR using genomic DNA from the carcasses as substrate. (C) Comparative immunolocalization of NAP1 protein (green) in the follicle cell layer surrounding egg chambers of wild-type and *Nap1* knockout mutant ovarioles. DNA is stained with propidium iodide (red). Egg chambers were stained with Alexa488-labeled phalloidin to reveal actin-rich structures (blue). (D) The same antibody applied to the follicle cell layer of egg chambers from a wild-type and a *Nap1* knockout mutant fly. Actin (here, red) reveals the apical part of the follicle cells. In the knockout mutant the basal concentration of NAP1 protein (green) observed in wild-type cells is absent (arrowheads).

Drosophila has suffered so far from the lack of an equally efficient gene-targeting method. Only recently a promising method was established (RONG and GOLIC 2000, 2001; RONG *et al.* 2002). The results presented here provide added support for the efficacy of this targeting technique in Drosophila and for its applicability to any arbitrary locus. The study had three subgoals: (1) screening and verification of a targeted knockout event at the *Nap1* locus, (2) a partial functional analysis of *Nap1*, and (3) examination of recombination tracts for an initial understanding of the underlying DNA repair pathway.

Generation of targeted *Nap1* mutant alleles: A mutant null allele for the Drosophila *Nap1* gene has not been available so far. Starting from genomic DNA of Canton-S wild-type flies, we used recombinant PCR (HIGUCHI 1990) to generate a mutagenized 4.3-kb *Nap1*-containing fragment holding all knockout mutations and tract markers. The altered *Nap1* fragment was subsequently cloned into the pTV2 vector (RONG *et al.* 2002) and transformed into the germline of w^{67c23} embryos. We found that starting from genomic DNA substantially reduces both the time and the cost associated with traditional library screening methods and it is at least as efficient as the construction of mouse-targeting vectors using methods such as recombination protein E (RecE) and RecT-mediated cloning (ZHANG *et al.* 1998, 2002). We then used the ends-in method of gene targeting (RONG and GOLIC 2000) to disrupt the *Nap1* locus. In our experiments, the targeting efficiency was dependent on the experimental conditions. The three screens used an identical donor insertion on a third chromosome balancer. Variations of the heat-shock conditions resulted in a significant improvement (Table 1). While the initial screen was not successful (no targeting event in 1100 single crosses with 165,000 scored chromosomes), the final screen was very efficient (five targeting events in 450 single crosses with 80,300 scored chromosomes; Table 1) This efficiency (~1 in 16,000 gametes) matched the reported efficiencies (~1 in 500 gametes to ~1 in 30,000 gametes) of RONG *et al.* (2002). We are not sure about the reasons for the initial failure, but, as we used an uncommon brand of thick-walled glass bottles for culturing flies, the duration (1 hr) of the initially applied heat shock may not have been sufficient to produce enough donor-construct excisions. Extending the duration of the heat shock and repeated heat shocks increased eye-color mosaicism and led to the targeted products (Table 1).

In addition to the six targeted events captured in our screen, we found only two donor integrations that did not map to the *Nap1* locus (at polytene-chromosome map position 60A of the second chromosome) but did map to the X chromosome. Neither of these integrations were targeted events at the endogenous *white* locus caused by homologous recombination with donor-internal *white* sequences. Targeting in mouse ES cells is often accompanied by high ratios of nontargeted insertions. Actually, when positive-negative selection is not used in ES cell transformation, the bulk of positively selected ES cell clones contain nontargeted insertions outnumbering targeted events by orders of magnitude (MANSOUR *et al.* 1988; BOLLAG *et al.* 1989). For Drosophila, RONG *et al.* (2002) report that in their screens the majority of positively selected (red eye-color marker) flies were targeted. Our results are consistent with this.

A drawback of the insertional targeting procedure (also called "knock-in targeting") is that the mutated gene as well as upstream and downstream regulatory sequences are duplicated. This might affect gene function in an unpredictable manner. Phenotypic and functional analysis of a targeted gene may be impaired fur-

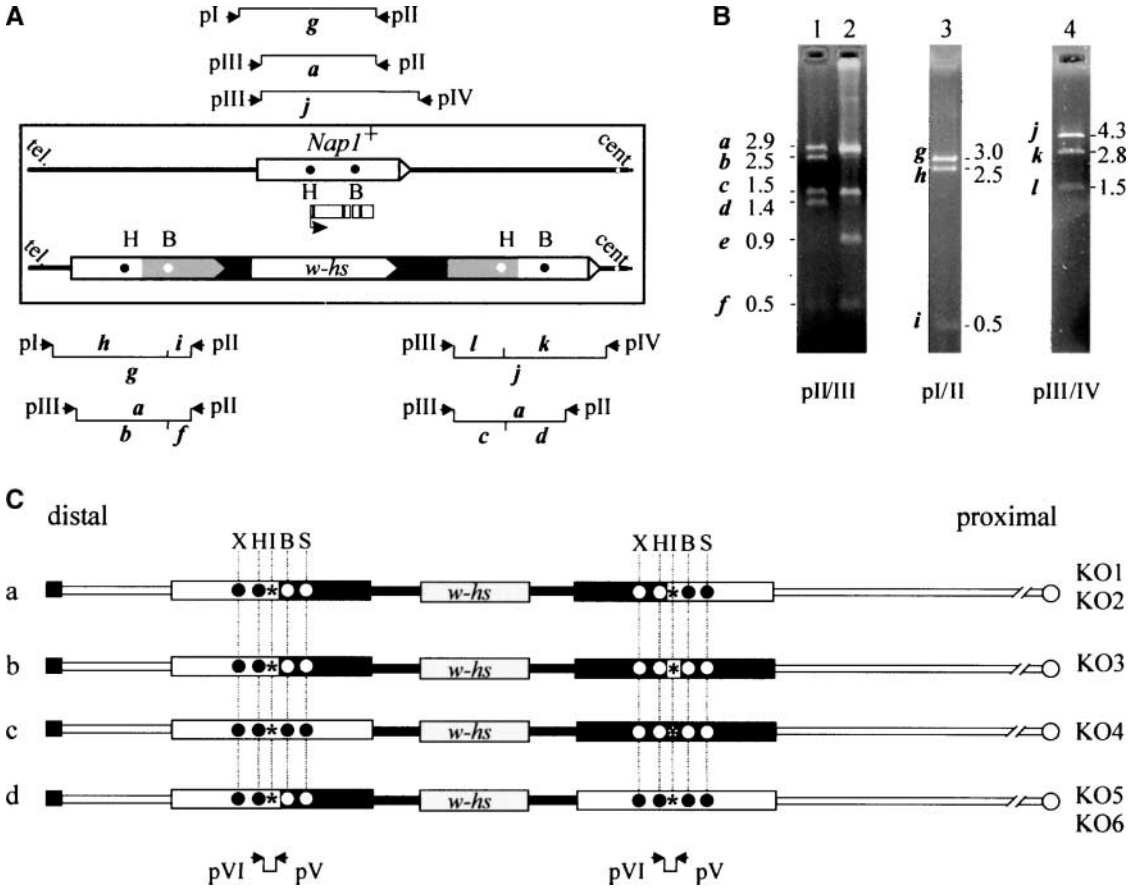

FIGURE 4.—Recombination tract analysis. (A) PCR analysis of genomic DNA from heterozygous recombinant KO flies. The boxed area shows the predicted genomic structure of two alleles of the *Nap1* locus. The top map designates the wild-type *Nap1* allele, the bottom map designates a full knockout allele. *w-hs* represents the *white-hs* positive selection marker gene. H and B designate the restriction enzymes *Hin*dIII and *Bcl*I. Open circles indicate the presence and solid circles the absence of these sites. Diagnostic primer combinations (pI–pIV) are outside of the boxed area. The expected PCR products and their restriction digest products using *Bcl*I and *Hin*dIII are indicated. Italic letters (*a–l*) indicate restriction fragments corresponding to sizes in B. (B) Detection of all predicted fragments. Here, only results for $Nap1^{KO1}$ are shown. Italic letters correspond to predicted fragment sizes in A. The internal structure of *Nap1*-targeting events was confirmed by a *Bcl*I/*Hin*dIII double digest (lane 1); lane 2 is a control from a fly containing the *Nap1* donor construct on the third chromosome. The 0.9-kb fragment (fragment *e* in B) diagnostic for the donor construct is missing in all KO flies (except KO4 where it derives from the proximal *Nap1* duplication). The distal region of the knockout rearrangement was diagnosed using *Bcl*I (lane 3, fragments *h* and *i*) and the proximal region was confirmed using *Hin*dIII (lane 4, fragments *k* and *l*). (C) Structure and recombination tracts of six knockout events at the *Nap1* locus. Recombination tract analysis was performed as described in A and B. Restriction enzyme markers were introduced into the donor vector, which are reciprocal to the wild-type *Nap1* target gene (X, *Xho*I; H, *Hin*dIII; I, *I-Sce*I; B, *Bcl*I; S, *Sal*I). Markers correspond to wild-type (solid circle) and mutagenized (open circle) *Nap1* sequences. Presence (open star) or absence (solid star) of the *I-Sce*I cutting site is indicated. Primer combinations pV and pVI were used to amplify each *I-Sce*I site for subsequent sequencing. (a) Knockout alleles $Nap1^{KO1}$ and $Nap1^{KO2}$ revealed identical recombination tracts. These recombination products represent the predicted knockout structure. (b) $Nap1^{KO3}$ represents the third complete knockout event. The proximal duplication is completely derived from the donor construct except for the *I-Sce*I cutting sequence. (c) Incomplete knockout allele $Nap1^{KO4}$. The distal duplication is wild type. The proximal duplication is identical to the donor construct. (d) $Nap1^{KO5}$ and $Nap1^{KO6}$ represent partial knockout alleles. The proximal duplication is entirely wild type.

ther if the gene is small (*e.g.*, 1 kb) and tightly flanked by neighboring genes. Because the donor sequence should be long (*e.g.*, 4–5 kb) for homology requirements during recombinational DNA repair, it cannot always be avoided that the flanking genes are duplicated as well. This would make functional studies unreliable. These difficulties are now overcome by the use of a meganuclease cutting site (*I-CreI*) within the integrated pTV2-vector (RONG *et al.* 2002). A DSB can now be produced between the two copies of the duplicated target gene, and the DSB is repaired through single-strand annealing (SSA) repair such that a single copy of the targeted gene remains. The whole procedure therefore resembles a "hit-and-run" approach and satisfies the highest standards of gene targeting (ADAIR and NAIRN 1995).

A partial functional analysis of NAP1: Because the *Nap1* gene is large, the donor did not possess additional

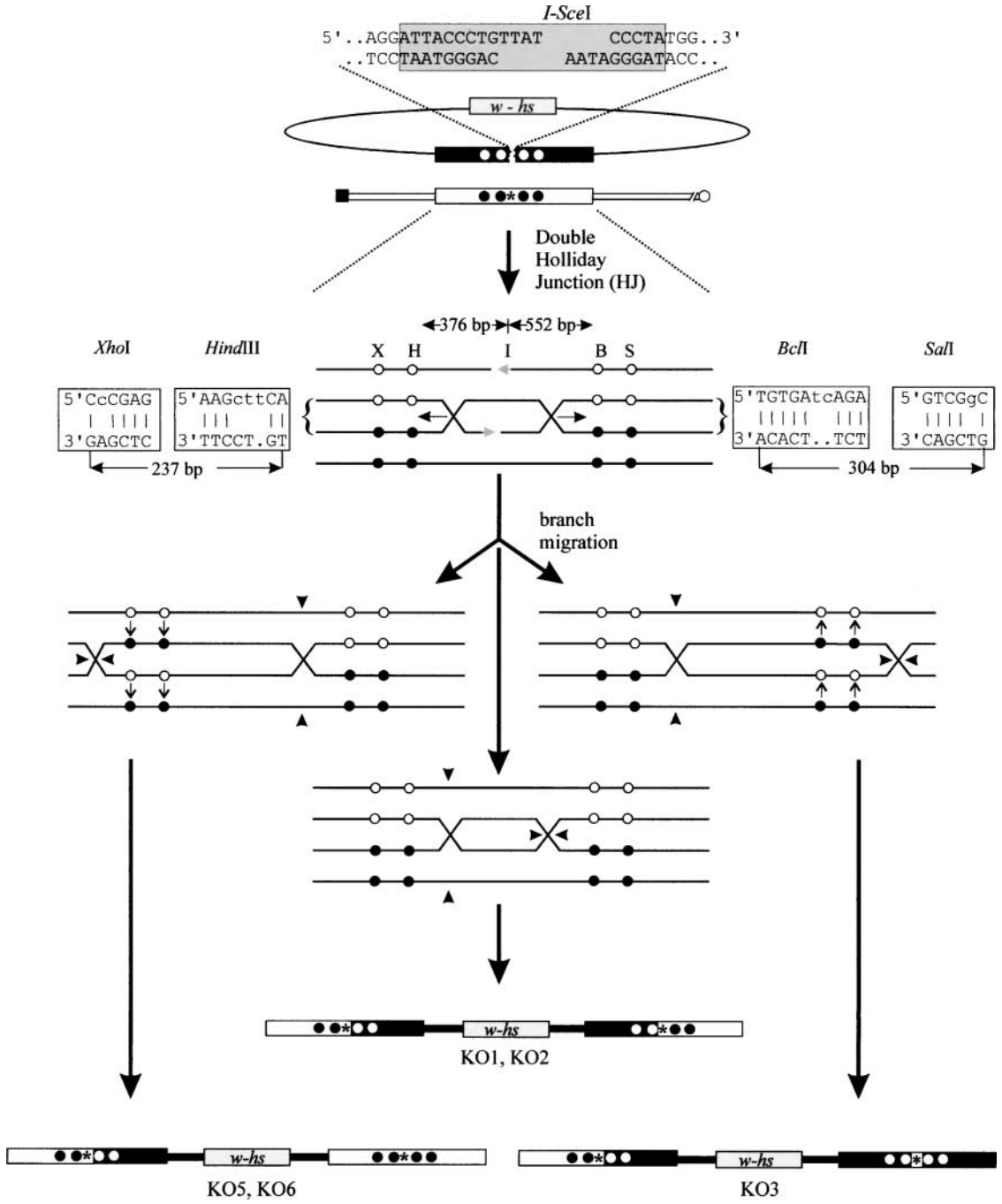

FIGURE 5.—Recombination tract data as interpreted using the classical DSB repair model (SZOSTAK *et al.* 1983). (Top) The initial presynaptic phase of DSB repair. The episome carries the *white-hs* positive selection marker (*w-hs*) and an *I-Sce*I meganuclease induced DSB centered within an altered *Nap1* gene. The chromosomal wild-type *Nap1* target is shown below. For proper DSB repair, exonuclease processing of the *I-Sce*I sequence is obligatory. (Middle) The synaptic and postsynaptic phases of DSB repair (for review see LANKENAU 1995). Shaded arrows represent the 3′ OH ends of the processed *I-Sce*I cut and the direction of DNA synthesis. After formation of Holliday junctions, branch migration (outward-pointing small black arrows) creates alternative heteroduplex strands. Mismatched sequences of tract markers are shown (boxed) with their relative distances to each other and to the *I-Sce*I cutting site. The observed conversion tracts of the *Nap1*-targeting products can be explained if mismatches are fixed in the direction shown by small open arrows. Resolution of Holliday junctions is indicated (arrowheads). (Bottom) The obtained knockout recombinants KO1, KO2, KO3, KO5, and KO6. The solid star indicates the absence of the *I-Sce*I site. A possible mechanism explaining why KO4 is compatible with the classical DSB model is shown in Figure 6.

genes whose altered expression pattern might affect a functional analysis of *Nap1*. Homozygous *Nap1* flies did not express detectable amounts of NAP1 protein (Figure 3). We found that first-generation homozygous mutant *Nap1* flies (derived from heterozygous parents) developed until the adult stage, albeit at sub-Mendelian

frequencies. These flies showed reduced viability, but they were weakly fertile and gave rise to a second generation of homozygous flies. In these flies, the phenotype became much stronger and more penetrant. The few escaper flies that developed to the adult stage showed impaired development and died a few days after eclosion. A functionally strong maternal component of *Nap1* expression at low concentrations (undetectable by Western blot) is probably sufficient to sustain relatively normal development in a significant fraction of homozygous mutant flies derived from heterozygous parents. Only after depletion of the maternally supplied components does the lethal phenotype become fully penetrant. The lethal phenotypes therefore were similar to the phenotype of other gene products thought to be important in nucleosome remodeling. For example, *imitation switch* (ISWI) homozygotes, where ISWI is the catalytic subunit of three essential chromatin-remodeling complexes NURF, ACF, and CHRAC, die as late larvae or early pupae (Varga-Weisz and Becker 1998; Deuring *et al.* 2000). The *Nap1* knockout mutants may therefore point toward related functions of NAP1.

Recombination tract analysis: In this study we engineered frameshift point mutations within the coding sequence of the *Nap1* gene, which blocked protein expression in the three knockout alleles $Nap1^{KO1}$, $Nap1^{KO2}$, and $Nap1^{KO3}$. Simultaneously these mutations served as markers so that we could monitor conversion tracts in the recombination products over a sequence distance of 1.5 kb. Because ends-in targeting generates a duplication of the donor DNA at the target locus, all markers are represented twice in a given targeting product. With the aid of the markers we identified four different types of recombinant flies among the six targeted recombination events that needed explanation (Figure 4C): Ends-in targeting is triggered by a linearized donor episome, whose DNA ends are sensed by the cell as double-strand DNA damage. The artificial, *I-Sce*I-induced break is thought to stimulate the DNA repair machinery of the cell. The donor construct contained the *I-Sce*I cutting sequence located in the center of the *Nap1* gene. Only one of the eight individual recombinant donor duplicates (Figure 4C) still contained an intact *I-Sce*I site ($Nap1^{KO4}$), but not a single nucleotide of the *I-Sce*I recognition sequence was found in the other seven copies. This result indicated that cellular exonucleolytic activities enlarge the DSB made by *I-Sce*I at least beyond the length of this sequence (18 bp). Both single-strand DNA as well as dsDNA from the staggered *I-Sce*I site cut were removed. This necessitated some trimming activity of both the 3′ ssDNA overhang and the 5′ double-strand end of the break. Resection of ends is known to occur by 5′-to-3′ exonucleases or by an endonuclease associated with a helicase producing long 3′-ended tails (Paques and Haber 1999). The tails then are thought to invade a homologous template. In the course of DSB repair the gap is finally restored by DNA replication initiated at the invaded 3′ ends (Szostak *et al.* 1983). Rong *et al.* (2002) describe the practical implications of this exonuclease activity. They successfully introduced mutations to the target genes 400–1300 bp from the *I-Sce*I site. In agreement with this, the marker positions in our experiments were located 376 bp (*Hin*dIII), 552 bp (*Bcl*I), 613 bp (*Xho*I), and 856 bp (*Sal*I) from the DSB.

How do the recombination tracts observed in the *Nap1* recombination products match known DSB repair pathways? Four prominent DSB repair pathways are relevant for this study: (1) nonhomologous end joining (NHEJ; Roth and Wilson 1988), (2) the classical DSB repair model (Szostak *et al.* 1983), (3) SDSA (Nassif *et al.* 1994), and (4) BIR (Malkova *et al.* 1996). A fifth DSB repair pathway, SSA, is important when a targeted knock-in duplication (as produced here) is transformed into a true targeted single-copy knockout mutation.

NHEJ is unlikely to play a role during targeted donor integrations. The most frequent DSB repair mechanism of metazoans, NHEJ was first recognized by Barbara McClintock in the early 1940s (McClintock 1987). It results in the ligation of broken DNA ends that share little or no homology with each other. This reaction is extremely efficient in eukaryotes, but the structures of the *Nap1* knockout alleles (Figure 4C) cannot be explained by NHEJ. End joining left remnants of the *I-Sce*I site behind, but after sequencing all target/donor joints we found only wild-type sequences at the *Nap1* donor-target junctions, except at the proximal *Nap1* copy of $Nap1^{KO4}$ where the *I-Sce*I site remained intact (Figure 4C, c). NHEJ, however, may account for the two nontargeted *Nap1* donor integrations on the X chromosome (Table 1).

Most likely, recombinational repair mechanisms are responsible for the targeted recombinants. Currently, the classical DSB repair model (Szostak *et al.* 1983) appears to be the most plausible explanation of the $Nap1^{KO}$ recombinants (Figure 4). Figure 5 gives details on the DSB repair model for some of the $Nap1^{KO}$ recombinants combined with the results of our tract analysis. The model readily explains the $Nap1^{KO1}$ and the $Nap1^{KO2}$ products by resolution of the double Holliday junctions before the tract markers have been copied by DNA synthesis or incorporated into heteroduplex. The tract patterns of $Nap1^{KO3}$, $Nap1^{KO5}$, and $Nap1^{KO6}$ can be explained readily as well if one assumes branch migration beyond one pair of diagnostic markers, followed by biased heteroduplex repair and subsequent branch resolution as indicated in Figure 5. The tract data encompass four diagnostic markers (excluding the nucleolytically processed *I-Sce*I site) in each duplicate of the six targeted $Nap1^{KO}$ products. Therefore, a total of 48 markers representing 24 tract marker pairs were analyzed. All pairs were continuous, which might not be a direct prediction of the "Szostak model." Biased strand-specific mismatch repair might account for this result (Figure 5). For example, meiotic recombination is believed to occur via

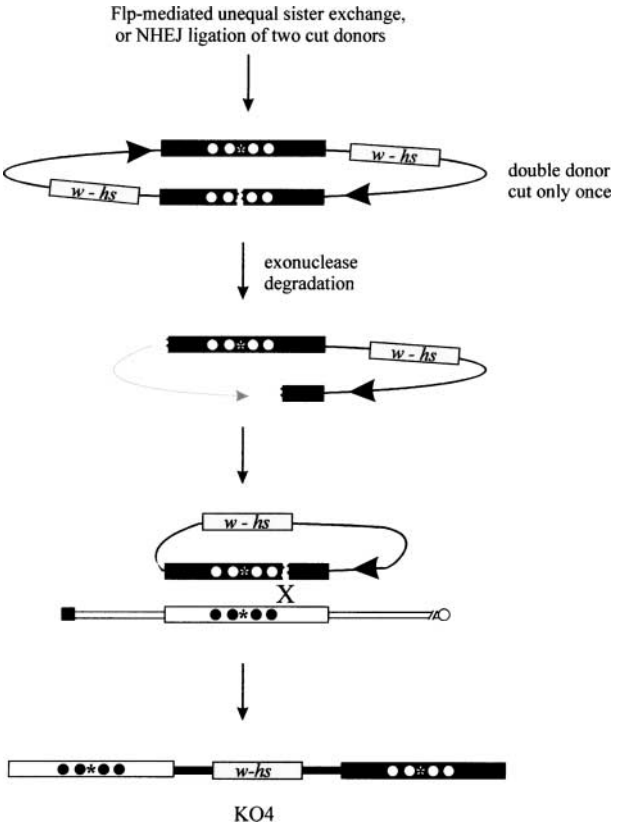

FIGURE 6.—Complex recombination events explaining KO4. Unequal crossing over between two FRT sites (arrowhead) or NHEJ ligation of two cut donors produces an episomal tandem donor array. Because *I-Sce*I cutting efficiency may be as low as 30% (WHITE and HABER 1990), only one of the *I-Sce*I sites within the donor produced a DSB. Resection of the ends and subsequent classical DSB repair (X) produced the targeted KO4 recombinant. Targeting involving two donors has been described (RONG and GOLIC 2000). Symbols are as described in Figure 4.

some version of the DSB repair model. Interestingly, it was reported that conversion tracts have always been continuous (CURTIS *et al.* 1989), hinting toward some kind of biased mismatch correction system. *Nap1*^{KO4} is the most difficult product to be explained by the classical model. This targeted recombinant still contained an intact *I-Sce*I recognition sequence. As the product is clearly a targeted recombination event, it could be explained by an endogenous DSB outside the region of the diagnostic markers followed by DSB repair. Alternatively, *Nap1*^{KO4} could be explained by fusion and subsequent trimming of two donor molecules followed by DSB repair, as indicated in Figure 6.

Are there alternative explanations for the targeted recombinants? A key question is whether the processed free ends of the linearized donor episome invade the target gene in a concerted manner, or whether both ends invade rather independently into template DNA. *P*-element-induced gap repair in Drosophila established that ectopic template DNA can be efficiently copied into target DNA located on a different chromosome (GLOOR *et al.* 1991; NASSIF *et al.* 1994; LANKENAU *et al.* 1996). Such events are not predicted by the conventional DSB repair model of Szostak (SZOSTAK *et al.* 1983). According to the SDSA model (NASSIF *et al.* 1994), the ends of the break independently undergo a genomewide homology search and it is thought that use of different, distantly located template sequences could be used for gene conversion (reviewed in LANKENAU 1995; LANKENAU and GLOOR 1998). This prediction was subsequently demonstrated in yeast. It was shown that a broken plasmid can acquire genetic information from two different loci on two different chromosomes (SILBERMAN and KUPIEC 1994). A similar experiment involved templates and targets, where each end of a DSB on a plasmid was homologous to one of two overlapping truncated genes (LEU2) on two different chromosomes. Restoration of an intact LEU2 gene was made possible only by two separate strand invasion events and the subsequent annealing of DNA ends (PAQUES *et al.* 1998). Interestingly, SDSA in Drosophila *P*-element-induced gap repair always resulted in nonreciprocal conversion between template DNA and target break (*i.e.*, the template sequence was never altered; GLOOR *et al.* 1991). This result indicates that SDSA is an improbable pathway for ends-in gene targeting because the invading strands of the linear donor episome would be finally unwound and returned to the broken strand (the linearized episome). Therefore, SDSA would not result in a targeted integration.

While SDSA is not the mechanism for targeted (knock-in) mutagenesis in Drosophila, the fact that the ends of a DSB undergo a genomewide homology search (ENGELS *et al.* 1994; LANKENAU *et al.* 2000) may hold true for the ends of the broken pTV2 episome as well. Analogous to SDSA, invasion of the episomal ends would lead to replication. However, instead of unwinding and returning to the broken strand, further DNA synthesis might pause in a true replication fork. This might be captured and resolved by an endogenous replication fork during the following S phase of the cell cycle. The process resembles BIR (as shown in Figure 7), but it would not be restricted to the telomere as originally proposed for yeast (MALKOVA *et al.* 1996; KRAUS *et al.* 2001). ENGELS (2000) proposed the mechanism for Drosophila partially to explain why targeting of the X-linked *yellow* gene was more efficient in females (with two X's) than in hemizygous males. This, however, is not a relevant argument for BIR as it is now reported that targeting of autosomal genes is also more efficient in females (RONG *et al.* 2002). Further, as the models shown in Figure 7 include exonuclease activity and template switching, they are at least as complicated as those that explain the targeted *Nap1* recombinants by classical DSB repair (Figures 5 and 6). However, the theoretical possibility of both broken ends invading different template strands may represent an option to test for the relevance

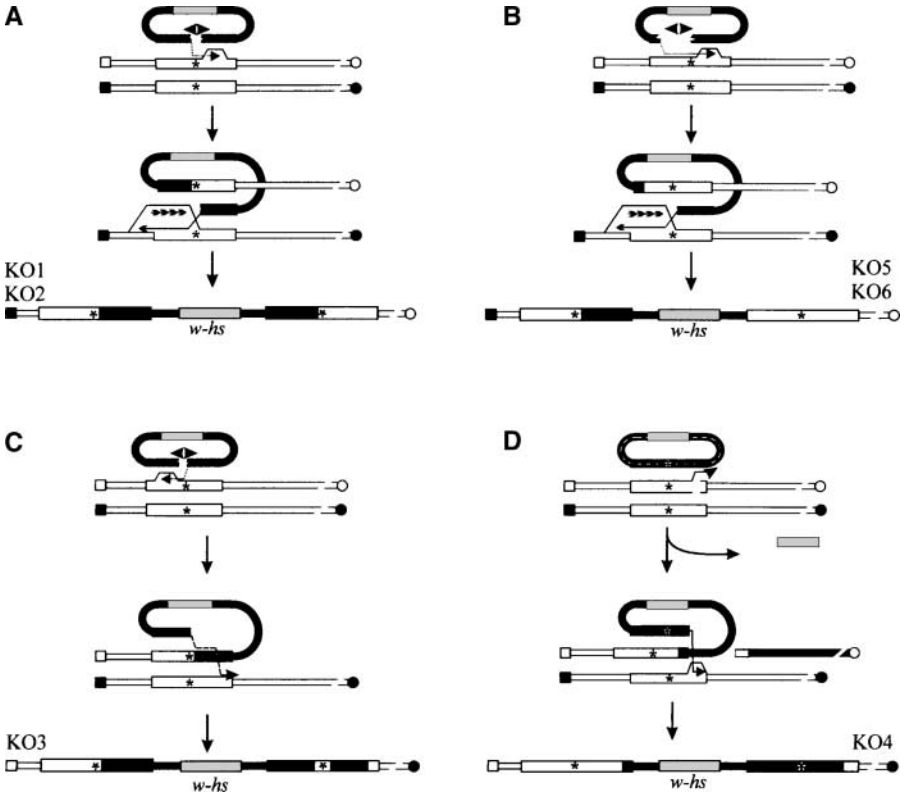

FIGURE 7.—Models based on BIR for the generation of four types of targeted recombination events. BIR can be initiated by strand invasion of the broken donor gene into the wild-type template gene (A–C) or by invasion of the endogenously broken template gene into the donor episome (D). Modifications in the length of processed broken ends (double arrow) and template switching determine the outcome of the targeted recombination. Solid and open stars as in Figure 2. KO1–KO6 represent the six targeted knockout alleles. *w-hs* (*white-hs*) designates the dominant marker insertion of the knockout duplications. Leading- and lagging-strand synthesis are indicated. (A) Production of true knockout products KO1 and KO2. Assumed minor exonuclease activity (not extending 376 and 552 bp left and right, respectively, to the I-site) removes no tract markers but the *I-Sce*I cutting sequence. (B) Extensive processing by assumed exonuclease activity removed all markers on the left side of the DSB but no marker on the right side. KO5 and KO6 represent incomplete knockout alleles. (C) KO3 represents a full knockout allele in which the left knockout copy is one-half wild type and one-half mutant. The right knockout copy, however, contained a full recombination tract except for the complete absence of the *I-Sce*I site. This allele can best be explained by template switching. (D) The presence of a complete repair tract in the KO4 allele, including the intact *I-Sce*I cutting site, is explained by an endogenous DSB in the target gene but not in the donor episome. Replication on the episome and subsequent template switching explained the right-hand knockout duplication while the left hand was wild type, indicating that the DSB occurred far to the right of the solid star in the wild-type gene.

of a BIR-like process. One experiment might be to test whether targeted knock-in recombinants are frequently associated with crossing over between homologous chromosomes. An extended analysis of long recombination tracts in targeted genes may also give further evidence in favor or against one of the pathways.

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