5′-CCCATTGTGACTCTACACCT-3′) followed by a Hinc II restriction indicated the presence of the point mutations in the homozygous mutants.
13. The immunoblot analysis was done with protein from adult brain with monoclonal antibodies to αCaMKII and synaptophysin (Boehringer). The secondary antibody was labeled with ^{125}I. Blots analyzed with an antibody to βCaMKII (Zymed Laboratories) showed that βCaMKII is expressed at normal amounts in adult brain of the αCaMKII$^{T286A\text{-}129B6F2}$ mutants. For the immunocytochemical analysis, coronal sections of the adult brain were incubated with the monoclonal antibody to αCaMKII. Antigen-antibody complexes were visualized with the glucose oxidase–diaminobenzidine-nickel method (*31*).
14. The kinase activity was determined from hippocampal slices with autocamtide-3 as substrate as previously described (*11*, *32*). For wild-type mice (n = 3), the total activity was 5.07 ± 0.91 pmol^{-1} μg^{-1} min, and the CaM-independent activity was 1.09 ± 0.12 pmol^{-1} μg^{-1} min (22.1 ± 1.8%). For the homozygous mutants (n = 3), the total activity was 4.74 ± 1.12 pmol^{-1} μg^{-1} min, and the CaM-independent activity was 0.40 ± 0.06 pmol^{-1} μg^{-1} min (9.2 ± 1.9%).
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16. Transverse hippocampal slices (400 μm) from 5- to 10-month-old mice were placed in a submerged recording chamber perfused continuously with artificial cerebrospinal fluid (ACSF) equilibrated with 95% O_2 and 5% CO_2 at 31°C. Extracellular fEPSPs were recorded with an electrode filled with ACSF in CA1 stratum radiatum, and the Schaffer collaterals were stimulated. A second pathway was used to control the stability of the recordings. The stimulus duration was 100 μs. The ACSF contained 120 mM NaCl, 3.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM D-glucose.
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19. Excitatory postsynaptic currents were recorded from CA1 pyramidal neurons from 6- to 12-month-old mice with a patch electrode (7 to 10 Mohm) in the whole-cell voltage-clamp mode. The pipette solution contained 122.5 mM cesium methanosulphonate, 17.5 mM CsCl, 10 mM Hepes buffer, 0.1 mM EGTA, 8 mM NaCl, 2 mM Mg–adenosine triphosphate, and 0.3 mM Na_3–guanosine triphosphate (pH 7.25, 290 to 300 mosm). A second pathway was used to control for the stability of the recordings. Picrotoxin (100 μM) was present in all experiments.
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25. Two- to five-month–old mice were studied in the spatial version of the water maze for 5 days (12 trials per day; blocks of four trials), as previously described (*33*). Transfer tests were given at the end of days 3 and 5. In another experiment, the mice were tested for 2 days with a visible platform in a fixed location (12 trials per day); then the visible platform was replaced by a hidden platform, and the mice were tested for another 2 days (12 trials per day). A transfer test was given at the end of training. Data were studied with one-way and two-way analysis of variance.
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Conjugative Transfer by the Virulence System of *Legionella pneumophila*

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Legionella pneumophila, the causative agent of Legionnaires' pneumonia, replicates within alveolar macrophages by preventing phagosome-lysosome fusion. Here, a large number of mutants called *dot* (defective for organelle trafficking) that were unable to replicate intracellularly because of an inability of the bacteria to alter the endocytic pathway of macrophages were isolated. The *dot* virulence genes encoded a large putative membrane complex that functioned as a secretion system that was able to transfer plasmid DNA from one cell to another.

A number of intracellular bacterial pathogens, such as *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, and *Legionella pneumophila*, grow within membrane-bound compartments diverted from the normal endocytic pathway of host cells (*1*). *Legionella pneumophila* replicates within alveolar macrophages by preventing acidification of the nascent phagosome and subsequent fusion with lysosomes (*2*). Several *L. pneumophila* genes (*dotA* and *icmWXYZ*) that are required for growth in macrophages have been identified (*3*). Mutations in these genes allow bacteria to be internalized into compartments that fuse with lysosomal components (*3*, *4*).

To understand how this organism prevents phagosome-lysosome fusion, we isolated a large collection of additional mutants that were defective for intracellular growth. Twenty-six spontaneous mutants were isolated on the basis of the observation that *L. pneumophila* strains resistant to low amounts of sodium chloride are often unable to replicate in macrophages (*5*, *6*). We independently isolated six additional mutants by screening mutagenized *L. pneumophila* for a lack of intracellular growth (*7*). Complementation of these mutants revealed two 20-kb regions on the *L. pneumophila* chromosome that contain a large number of genes required for growth in macrophages (Fig. 1). Region I contains three genes, *dotDCB*, located about 10 kb from the previously identified *dotA-icmWXYZ* locus. Region II contains 11 genes in three potential operons (*dotML*, *dotKJIHGFE*, and *dotNO*). The majority of the *dot* and *icm* genes identified to date, 14 of 19, are predicted to encode proteins that are membrane-associated. Although most of these proteins are not homologous to any known open reading frames (ORFs), four Dot proteins have limited homology to components of bacterial conjugation systems (Fig. 1). The COOH-terminus of DotG is homologous to Trb I, a protein required for conjugation of the IncP plasmid RP4 (23% identity over the COOH-terminal 442 amino acids of DotG) (Fig. 1) (*8*). DotM and DotL have homology to TrbA and TrbC, respectively, from the Inc I plasmid R64 (23% identity for DotM and TrbA and 26% identity for DotL and TrbC)

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(9). Finally, DotB is homologous to a large family of nucleotide-binding proteins (for example, TrbB) that includes members of various conjugal-transfer systems (6).

To examine the role of one of the *dot* genes with homology to conjugation genes, we constructed a large in-frame deletion in *dot*G (*10*). The *dot*G deletion mutant was assayed for survival and replication in the monocytic cell line U937, which supports intracellular growth of virulent *L. pneumophila* (*11*). Wild-type *L. pneumophila* containing the vector pKB5 showed an increase in viable counts of 10^3 to 10^4 cells in 72 hours, whereas the *dot*G mutant with the same vector showed no growth during this time frame (Fig. 2A). Introduction of a plasmid encoding the *dot*G ORF (*12*) restored growth of the *dot*G deletion mutant to wild-type amounts. The *dot*G mutant was also defective in altering the endocytic pathway, as exhibited by colocalization of phagosomes containing the *dot*G mutants and a late endocytic marker, LAMP-1 (Fig. 2B). In contrast, wild-type *L. pneumophila* are normally able to prevent phagosome-lysosome fusion, and therefore phagosomes containing these bacteria are relatively devoid of endocytic and lysosomal marker proteins (*2*, *13*). This lack of proteins can be seen by a lack of colocalization of wild-type *L. pneumophila* phagosomes with LAMP-1 (Fig. 2B) (*13*). The *dot*G mutant showed a targeting defect similar in magnitude to that of a previously characterized *dot*A mutant (80% LAMP-1 positive) in comparison with wild-type cells (20% LAMP-1 positive) (*3*). Thus, loss of a *dot* gene homologous to a conjugation gene resulted in a failure to replicate intracellularly because of an inability to alter targeting similar to that seen with the original *dot*A-*icm*WXYZ mutants (*3*, *4*).

To test if the homology of DotG to a protein required for conjugation was relevant, we attempted to determine if the Dot proteins could have evolved from a DNA transfer system. We assayed transfer of the mobilizable IncQ plasmid RSF1010, which codes for products involved in conjugative DNA processing but lacks the proteins involved in conjugal-pair formation and therefore requires those functions to be provided in trans (*14*). *Legionella pneumophila* was able to mobilize RSF1010 to another strain of *L. pneumophila* at a frequency of about 10^{-6} conjugants per donor (Table 1, top). *Legionella pneumophila* was also able to transfer RSF1010 to two different strains of *Escherichia coli* (ER1793 and MM294) at about the same rate (*15*). Transfer required a cis-acting site on the plasmid, the origin of transfer (*oriT*), as normally seen with conjugation (Δ*oriT* in Table 1, top) (*14*). Moreover, the presence of deoxyribonuclease I (DNase I) had no effect, indicating that mobilization was not due to transformation by free DNA. Transfer required functional DotG protein because the Δ*dot*G strain characterized above was unable to transfer the RSF1010 plasmid pKB5, whereas providing the *dot*G ORF on pKB5 restored transfer to wild-type amounts (Table 1, bottom). Transfer also required

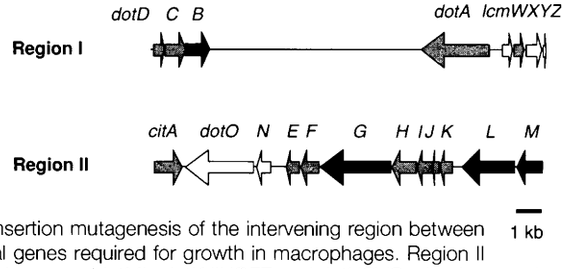
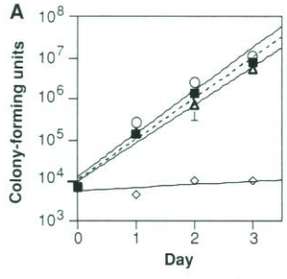


Fig. 1. *Legionella pneumophila* genes required for growth in macrophages. *dot* genes are located in two regions, I and II, each consisting of about 20 kb of DNA. *dotDCB* (GenBank accession number AF026533) is located 10 kb from a previously identified locus required for intracellular growth consisting of the genes *dotA* and *icmWXYZ* (*3*). Insertion mutagenesis of the intervening region between *dotA* and *dotB* revealed no additional genes required for growth in macrophages. Region II contains 11 *dot* genes in three potential operons (*dotML*, *dotKJIHGFE*, and *dotNO*) (Genbank accession number AF026534). Region II is flanked on one side by the *L. pneumophila* homolog of *citA*, a plasmid-encoded citrate-proton symporter found in certain *cit*$^+$ *E. coli* strains (*21*). *dotB*, *dotE*, and *dotG* were identified by the salt selection (*6*), *dotH* and *dotO* by the mutagenesis screen (*7*), and the remaining genes by sequencing the surrounding region. Proteins encoding the genes that are shaded black have homology to known conjugation proteins, and proteins encoding the genes that are shaded gray are predicted to be membrane associated.



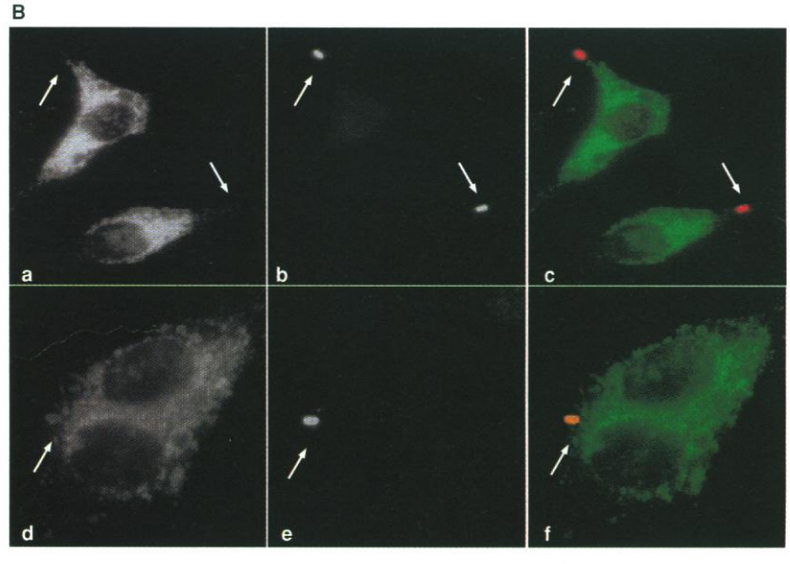


Fig. 2. (**A**) DotG is required for intracellular growth. Phorbol ester–transformed U937 cells were challenged with a multiplicity of between 1 and 10 bacteria per macrophage, and growth was assayed as previously described (*3*). Samples from individual microtiter wells were taken daily in triplicate, and viable counts were titered on bacteriological plates. Growth was assayed for Lp02/pKB5 (○), a wild-type strain containing the vector pKB5 (*3*); Lp02/pJB480 (△), a wild-type strain containing a plasmid expressing DotG$^+$ (*15*); JV573/pKB5 (◇), a Δ*dotG* mutant strain with vector pKB5 (*13*); and JV573/pJB480 (■), a Δ*dotG* mutant strain with DotG$^+$ ORF. (**B**) Phagosomes containing the *dotG* mutant colocalize with the late endocytic marker LAMP-1. Mouse bone marrow macrophages were challenged at a multiplicity of infection between 1 and 10 bacteria per macrophage to determine the intracellular localization of strains lacking functional *dotG*. After a 1-hour incubation at 37°, samples were fixed, permeabilized, and stained with antibodies to *L. pneumophila* to localize intracellular bacteria and antibody to LAMP-1 to stain the endocytic compartment (*16*). The top panels (a to c) are the wild-type *L. pneumophila* strain Lp02, and the bottom panels (d to f) are the *dotG* mutant strain JV573. LAMP-1 staining is shown in (a) and (d), *L. pneumophila* staining in (b) and (e), and a composite of the LAMP-1 and bacterial staining in (c) and (f), consisting of LAMP-1 in green, *L. pneumophila* in red, and areas of colocalization in orange. The colocalization was performed with Color Merge of IP-Lab Spectrum (Signal Analytics, Vienna, Virginia). The efficiency of intracellular trafficking was assayed by quantification of the number of phagosomes containing bacteria that colocalized with LAMP-1 ($n > 200$ phagosomes assayed in three separate experiments).

the donor strain to have a functional copy of *dotB*, one of the other genes with homology to a conjugation gene. In addition, mobilization was dependent on genes with no homologies to known conjugation genes, including the previously characterized *dot*A and *icmWXYZ* genes as well as one of the genes we identified, *dotE*.

Because the Dot proteins are capable of mobilizing RSF1010, they likely constitute a secretion system that is capable of transferring a substrate across the outer membrane. During intracellular growth, this system could deposit a factor or inhibitor into macrophages to subvert the endocytic pathway. *Legionella pneumophila* may have acquired the *dot* secretion system by adaptation of the conjugation system of an integrated plasmid and may be another example of a pathogenicity island (*16*).

The *L. pneumophila dot* virulence system may be distantly related to specialized secretion systems, termed type IV, found in several other pathogens (*17*). *Agrobacterium tumefaciens* contains an operon of 12 genes, *virB*, which has extensive homology to a traditional plasmid transfer system and is used to inject oncogenic transferred DNA into plant cells (*18*). *Agrobacterium tumefaciens* is also able to transfer RSF1010 plasmid from one cell to another (*19*). *Bordetella pertussis* contains a related operon, *ptl*, which is used to secrete pertussis toxin (*20*). In contrast to these systems, *L. pneumophila* contains only two conserved proteins (DotB has homology to VirB11 and DotG has homology to VirB10). Moreover, these genes are not found in a single large operon of multiple conjugation genes as seen with the *virB* and *ptl* operons (*17*).

The actual substrate transferred by *L. pneumophila* into macrophages is presently unknown. However, it would seem unlikely that it injects "pathogenic" DNA into mammalian cells early in infection as *A. tumefaciens* does to plant cells because the endocytic pathway is altered extremely rapidly within minutes of uptake (*4*). In contrast, it is more likely that *L. pneumophila* transfers a protein that acts as an inhibitor or modifier of the endocytic pathway. The discovery that *L. pneumophila dot* genes are likely to form a secretion machinery provides the first functional indication of how *L. pneumophila* subverts the endocytic pathway of a macrophage. Understanding how this pathogen exploits a conjugal-transfer system for intracellular growth may shed light on how other clinically important pathogens, such as *Chlamydia* and *Mycobacterium*, cause disease.

Note added in proof: After completion of the refereeing of this manuscript, Segal and Shuman (*22*) reported *icm*O and *icm*P, which are identical to *dot*L and *dot*M, and indicated the presence of conjugal transfer.

Table 1. The *L. pneumophila dot* virulence loci are required to transfer RSF1010 plasmid into recipient bacteria. Mating was assayed by mixing 1.0×10^9 *L. pneumophila* containing noted plasmids with a tenfold excess of a recipient bacteria strain. Matings were performed in triplicate by allowing the mixed bacterial cultures to incubate for 2 hours at 37°C on 45-mm Millipore hemagglutinin filters (HAWP 047 S0) placed onto prewarmed charcoal-yeast extract media with thymidine (CYET) plates (*3*).

Donor strain*	Media†	Recipient‡	Number of conjugants per donor§
RSF1010	CYET	Lp01	3.9×10^{-6}
RSF1010	CYET	*E. coli* ER1793	6.6×10^{-7}
RSF1010	CYET	*E. coli* MM294	2.2×10^{-7}
RSF1010	CYET + DNase I	Lp01	3.2×10^{-6}
RSF1010	CYET + DNase I	*E. coli* ER1793	9.1×10^{-7}
RSF1010	CYET + DNase I	*E. coli* MM294	2.4×10^{-7}
RSF1010 Δ*oriT*	CYET	Lp01	$< 3.8 \times 10^{-9}$
RSF1010 Δ*oriT*	CYET	*E. coli* ER1793	$< 4.0 \times 10^{-9}$
RSF1010 Δ*oriT*	CYET	*E. coli* MM294	$< 3.8 \times 10^{-9}$
RSF1010	CYET	*E. coli* C600	$< 3.7 \times 10^{-9}$

Donor strain‖	Donor plasmid¶	Recipient	Number of conjugants per donor#
Wild type	pKB5	*E. coli* MM294	2.5×10^{-6}
dotG$^-$	pKB5	*E. coli* MM294	$< 4.3 \times 10^{-9}$
dotG$^-$	p*dotG*$^+$	*E. coli* MM294	3.5×10^{-6}
dotB$^-$	pKB5	*E. coli* MM294	$< 5.6 \times 10^{-9}$
dotB$^-$	p*dotB*$^+$	*E. coli* MM294	4.8×10^{-6}
dotA$^-$	pKB5	*E. coli* MM294	$< 6.1 \times 10^{-9}$
dotA$^-$	p*dotA*$^+$	*E. coli* MM294	9.2×10^{-6}
icmWXYZ$^-$	pKB5	*E. coli* MM294	$< 1.0 \times 10^{-8}$
icmWXYZ$^-$	p*icmWXYZ*$^+$	*E. coli* MM294	3.4×10^{-6}
dotE$^-$	pKB5	*E. coli* MM294	$< 5.8 \times 10^{-9}$
dotE$^-$	p*dotE*$^+$	*E. coli* MM294	8.7×10^{-6}

*Lp02, a replication-competent strain (*3*), was transformed by electroporation with either RSF1010 Kan, an RSF1010 plasmid containing kanamycin from Tn903 (RSF1010 in table) (*18*), or RSF1010 Δ*oriT*, an RSF1010 plasmid containing a deletion (Δ13) in the origin of transfer (*oriT*) that completely abolishes conjugation (*18*). †Matings were performed on CYET or CYET containing DNase I (1 μg/ml). ‡Recipients were either a *L. pneumophila* strain competent for intracellular growth (Lp01) (*3*), the restriction minus *E. coli* strains ER1793 (*hsdR*) and MM294 (*hsdR*), or the restriction-competent *E. coli* strain C600 (*19*). §*Legionella pneumophila* conjugants were selected on charcoal-yeast extract lacking thymidine to select against the thymine auxotrophic donor Lp02, as well as kanamycin at 20 μg/ml to select for plasmid transfer. *Escherichia coli* conjugants were selected on LB plates containing kanamycin at 25 μg/ml. The *L. pneumophila* donor strains are unable to grow on LB plates. ‖The donor strains are Lp02 (wild type), the *dotG* deletion strain (JV573), and the following four salt-resistant mutants (*6*): JV303 (*dotB*), JV309 (*dotA*), JV312 (*icmWXYZ*), and JV328 (*dotE*). ¶The donor plasmids were either the vector pKB5, an RSF1010 plasmid harboring Amp^R (*3*), or pKB5 containing the complementing ORFs for the various mutants. #*Escherichia coli* conjugants were selected on LB plates containing ampicillin (150 μg/ml). Reversion rates of markers used to select conjugants were substantially below the rates of transfer detected (for example, Lp02 + RSF1010 donor, $< 9.1 \times 10^{-11}$; Lp01 as a recipient, $< 7.7 \times 10^{-11}$; *E. coli* ER1793 as a recipient, $< 1.6 \times 10^{-11}$; and *E. coli* MM294 as a recipient, $< 1.1 \times 10^{-10}$).

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Meiotic Synapsis in the Absence of Recombination

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Although in *Saccharomyces cerevisiae* the initiation of meiotic recombination, as indicated by double-strand break formation, appears to be functionally linked to the initiation of synapsis, meiotic chromosome synapsis in *Drosophila* females occurs in the absence of meiotic exchange. Electron microscopy of oocytes from females homozygous for either of two meiotic mutants (*mei-W68* and *mei-P22*), which eliminate both meiotic crossing over and gene conversion, revealed normal synaptonemal complex formation. Thus, synapsis in *Drosophila* is independent of meiotic recombination, consistent with a model in which synapsis is required for the initiation of meiotic recombination. Furthermore, the basic processes of early meiosis may have different functional or temporal relations, or both, in yeast and *Drosophila*.

In the classical view of meiosis, homologous chromosome synapsis, as indicated by the formation of an elaborate ribbonlike structure called the synaptonemal complex (SC), was thought to be the first and primary event of meiotic prophase, essential for the initiation of meiotic recombination (*1*). Studies in *Saccharomyces cerevisiae*, however, have created a different view of the meiotic process in which the initiation of recombination, as evidenced by a double-strand break (DSB), precedes the initiation of synapsis (*2*, *3*). Three lines of evidence support this view of early meiotic prophase in yeast. First, the initiating event of meiotic recombination, the formation of a DSB, appears before SC formation (*4*). Second, meiotic mutants that either fail to create DSBs or to process DSBs to make single-stranded tails prevent the formation of a mature SC (*2*). Third, some mutants allow high levels of meiotic recombination but prevent the production of a mature SC (*5*). These data are consistent with a model in which single-stranded DNA generated by a DSB carries out a homology search required for synapsis and SC formation. In contrast, synapsis is not an absolute prerequisite for either the initiation (*6*) or completion of meiotic recombination (*7*).

To assess the relation between synapsis and the initiation of recombination in *Drosophila* oocytes, we examined both recombination and SC formation in oocytes homozygous for either of two null-recombination mutations. The *mei-W68* and *mei-P22* (*8*) mutants prevent the initiation of meiotic recombination as defined by four independent assays: (i) reduction or elimination of meiotic gene conversion; (ii) elimination of meiotic crossing over, as assayed by measuring either intragenic crossing over or the frequency of meiotic crossing over along entire chromosome arms; (iii) lack of double-strand DNA breaks that persist into metaphase or anaphase I; and (iv) failure to produce either early or late recombination nodules (RNs).

To assay the effects of the *mei-W68* and *mei-P22* mutations on meiotic crossing over, we examined intragenic recombination at the *rosy* locus (*9*). No gene conversion events or intragenic crossovers were observed among the progeny of *mei-W68* or *mei-P22* females (Table 1 and Fig. 1). Compared to controls, the frequency of both intragenic exchange and simple gene conversion was reduced by a factor of at least 30 to 40 and, most likely, was eliminated. A small reduction in gene conversion frequency was also observed in the *mei-W68*/+ females, suggesting a dosage effect.

The effect of these mutations on crossing over was also assayed by more conventional means. In both *mei-W68* and *mei-P22* mutant females, the frequency of crossing over along the entire X and second chromosomes was reduced to less than 0.5% of normal. Moreover, the few crossover events that were observed tended to be recovered in clusters of identical recombinants among the progeny of single females, suggesting that they resulted from mitotic and not meiotic recombination events (*10*). The failure to observe meiotic recombination events in progeny of *mei-W68* and *mei-P22* mutant

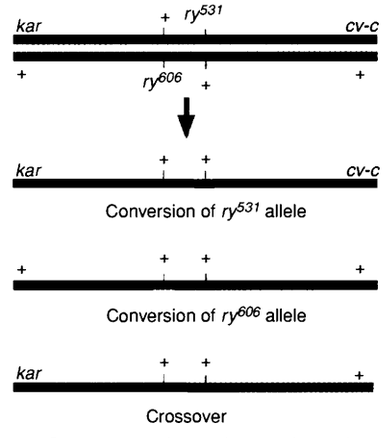

Fig. 1. Gene conversion at the *rosy* locus (genetic map position 52.0). A schematic of the parental chromosomes and the three products of intragenic recombination that yield *rosy*$^+$ recombinants. The distances between the loci are not drawn to scale. The recombinants were classified as convertants or crossovers on the basis of the flanking mutations. In the *mei-P22* experiment, the flanking markers were *kar*, an eye color mutant mapping 0.3 cM to the left, and *cv-c*, a wing vein mutant mapping 2.1 cM to the right of *ry*. In the *mei-W68* experiment, *Ace* (52.5) or *red* (53.6) replaced *cv-c* (54.1). The two *rosy* alleles used in this study, *ry*^{531} and *ry*^{606}, were chosen because they are at opposite ends of the *rosy* gene, 3780 nucleotides and 0.012 cM apart (*29*). The average length of conversion tracts in *Drosophila* is 885 base pairs (*32*), and therefore co-conversion events are expected to have a minimal effect on our experiments.

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