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Recurrent Locus-Specific Mutation Resulting From a Cryptic Ectopic Insertion in Neurospora

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ABSTRACT

New mutations are found among ~20% of progeny when one or both parents carry *eas* allele UCLA191 (*eas*^{UCLA}, *easily wettable*, hydrophobin-deficient, linkage group II). The mutations inactivate the wild-type allele of *cya-8* (*cytochrome aa*$_3$ *deficient*, linkage group VII), resulting in thin, "transparent" mycelial growth. Other *eas* alleles fail to produce *cya-8* mutant progeny. The recurrent *cya-8* mutations are attributed to repeat-induced point mutation (RIP) resulting from a duplicated copy of *cya-8*$^+$ that was inserted ectopically at *eas* when the UCLA191 mutation occurred. As expected for RIP, *eas*^{UCLA}-induced *cya-8* mutations occur during nuclear proliferation prior to karyogamy. When only one parent is *eas*^{UCLA}, the new mutations arise exclusively in *eas*^{UCLA} nuclei. Mutation of *cya-8* is suppressed when a long unlinked duplication is present. Stable *cya-8* mutations are effectively eliminated in crosses homozygous for *rid*, a recessive suppressor of RIP. The *eas*^{UCLA} allele is associated with a long paracentric inversion. A discontinuity is present in *eas*^{UCLA} DNA. The *eas* promoter is methylated in *cya-8* progeny of *eas*^{UCLA}, presumably by the spreading of methylation beyond the adjoining RIP-inactivated duplication. These findings support a model in which an ectopic insertion that created a mutation at the target site acts as a locus-specific mutator via RIP.

THE *easily wettable* gene was discovered when allele UCLA191 was obtained following mutagenesis of *Neurospora crassa* wild-type 74-OR8-1a using ethyl methanesulfonate (Selitrennikoff 1976). The gene has been of interest because of its physiological role in the cell, and this first allele has attracted special attention because of its curious genetic properities, described below. Whereas wild-type conidia are completely covered with a thin layer of hydrophobic rodlets, rodlets are absent in *eas* mutants (Beever and Dempsey 1978). Conidia of the mutant are hydrophilic, entering instantly into water suspension, in contrast to wild-type conidia, which are hydrophobic (Selitrennikoff 1976). Strains containing *eas*^{UCLA} are normal in growth rate, viability, and fertility. Linear growth of a typical strain continued at a normal rate through eight serial transfers on minimal medium at 34° in 30-cm growth tubes (method of Ryan *et al.* 1943), with no sign of senescence or stop–start growth when the experiment was terminated at 30 days.

David D. Perkins passed away on January 2, 2007. David made pivotal and unparalleled contributions to the Neurospora community and beyond. This article represents a typical collaborative effort that would not have seen the light of day without his exceptional dedication and talents.

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Cloning and sequencing of *eas*$^+$ was accomplished inadvertently. A circadian-clock-controlled gene originally called *ccg-2* (allele JD105; Bell-Pederson *et al.* 1992) and a blue-light-induced gene originally called *bli-7* (Lauter *et al.* 1992) were identified independently using messenger RNAs that were obtained under completely different conditions. Both of these mutations were first indicated to be *eas* alleles by DNA sequence similarity to the *rodletless* gene in Aspergillus (Stringer *et al.* 1991). Mutant alleles of *ccg-2* and *bli-7* both resembled *eas*^{UCLA} phenotypically and both mapped at the same locus (Bell-Pederson *et al.* 1992; Lauter *et al.* 1992).

The *eas*$^+$ gene encodes a cysteine-rich hydrophobic protein that is similar to hydrophobins identified in other fungi (Wessels *et al.* 1991; reviewed by Wessels 2000). In Neurospora, the hydrophobic rodlets of powdery wild-type conidia no doubt promote aerial dispersal in nature. In contrast, the hydrophilic rodlet-deficient conidia of *eas* mutants stick together and do not become airborne. Scoring of *eas* on agar slants is readily accomplished using a "tap test" to determine whether conidia shake loose or remain stuck together.

Experiments described in this study were initiated when an unexpected class of sparse-growing ("transparent") progeny was discovered in crosses parented by *easily wettable* allele UCLA191 (symbolized here as *eas*^{UCLA}). The anomalous progeny were suggested to result from recurrent mutation of the *cya-8* gene, which

is unlinked to *eas*, by virtue of repeat-induced point mutation (RIP; Selker 1990). Inactivation of genes by RIP in Neurospora had provided the first example of silencing when a duplicate copy of a DNA segment is added to the euploid genome (Selker *et al.* 1987; see Selker 1990, 2002 reviews). RIP has had a profound effect in shaping the Neurospora genome (Galagan *et al.* 2003; Galagan and Selker 2004). Compelling evidence indicates that RIP serves as a genome defense mechanism, providing protection from transposable elements (Selker *et al.* 2003). Here we describe a rearrangement that results in recurrent RIP-induced mutation. Evidence is presented that a gene that has been transposed to an ectopic locus can act as a mutator of its paralog in the original position. All evidence supports the hypothesis that the mutations are mediated by RIP, resulting from an ectopic copy of *cya-8* transposed to the *eas* locus.

MATERIALS AND METHODS

Strains: Standard Oak Ridge wild types were used, and markers were in Oak Ridge genetic background. Allele numbers are as follows: *ace-1*, Y2492; *adh* (*adherent*), NM227; *arg-12*, UM107; *cya-8*, P9178; *cyt-7*, 20; *fl* (*fluffy*), L; *nic-3*, Y31881; *pe* (*peach*), Y8743m; *rid-1*, N2250; *rip-1* (*ribosome production*), 4M; *trp-3*, td24; *un-15*, T54M50; *un-20*, P2402. The *helper-1* strain a^{m1}*ad-3B cyh-1* (FGSC 4564) was combined with *cya-8* strains to form phenotypically wild-type female-fertile heterokaryons. For information on markers and on the inactive mating-type *helper-1* strain, see Perkins (1984), Perkins *et al.* (2001), or http://www.bioinf.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.htm. Three mutant *eas* alleles were used: UCLA191 (eas^{UCLA}) (Selitrennikoff 1976), JD105 (eas^{JD}) (Bell-Pederson *et al.* 1992), and KH5-9 (eas^{KH}) (Hasanuma 1984). Translocations ALS179, OY329, and S1229 are described by Perkins (1997). Mutations at *cya-8* were not known previously. Existing stocks of eas^{UCLA} contain a wild-type linkage group (LG) VII with the $cya\text{-}8^{+}$ gene intact.

Genetic analysis: Crosses were made at 25° on slants of synthetic cross medium in 150-mm tubes. Ascospores were spread on 4% agar, isolated using a platinum–iridium blade to pick up single ascospores on a small piece of the underlying agar, transferred to 10 × 75-mm slants with appropriately supplemented Vogel's medium N, and heat-shocked 30 min in a 60° water bath. For media and general methods, see Davis and de Serres (1970) or http://www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm. When growth was not visible to the naked eye, tubes were examined for transparent growth using a dissecting microscope at ×40–×70 magnification with transmitted light from a substage mirror. For mapping *cya-8*, an exceptionally vigorous transparent strain (P9178, FGSC no. 4523) was used initially (as male parent—despite its vigor, the strain is female sterile). Because *cya-8* strains grow slowly and are female sterile, subsequent crosses employed phenotypically vigorous wild-type heterokaryons (FGSC 4524 A, 4525 a) combining a sparsely growing transparent strain with *helper-1* (a^{m1} *ad-3B cyh-1*) as the second component. The heterokaryons with *helper-1* are fully fertile either as female or as male, but the *helper* component does not participate in the cross because the a^{m1} mating-type allele is inactive (see Perkins 1984).

Each of the duplication strains used as a parent in Table 5 was obtained from a cross of the corresponding balanced translocation with wild type. In Neurospora, presence of a segmental duplication in one or both parents typically results in a cross being barren, *i.e.*, producing few or no ascospores (Raju and Perkins 1978). When a duplication was present, crosses were made by growing one parent on crossing medium in a petri dish and fertilizing the lawn with a conidial suspension. After perithecia had reached full size, the cross plates were inverted over an agar surface. Usually, a few ascospores were eventually ejected, and these were isolated not <30 days after fertilization. Because two or more spores often come from the same ascus, the numbers of progeny in Table 5 probably exceed the numbers of asci from which they originated.

Insertion of *fluffy* into the eas^{UCLA} inversion: The procedure was similar to that used by Bailey and Ebbole (1998) for obtaining a *fluffy* mutation in wild-type sequence by RIP mutagenesis, except that eas^{UCLA} strains were used instead of the Oak Ridge wild types. A plasmid (pFLUF3) that contains the fl^{+} gene on a 2.9-kb *Sma*I restriction fragment was used to transform eas^{UCLA} *mat A*. A transformant purified through three rounds of conidial passage was crossed to eas^{UCLA} *mat a*, and fast-growing *fluffy* strains were recovered among the progeny.

Molecular methods: To isolate genomic DNA, wild type (N150, 74-OR23-IVA), mutants eas^{UCLA} and eas^{JD}, and normal and slow-growing progeny of *eas* crosses were grown for 48 hr in Vogel's medium N. DNA was isolated as previously described (Miao *et al.* 2000). To detect DNA methylation by Southern analysis, ~1 μg of genomic DNA was digested with the cytosine methylation-sensitive *Sau*3AI endonuclease or its cytosine methylation-insensitive isoschizomer, *Dpn*II (Miao *et al.* 2000). Gel-purified probe fragments for Southern analysis were generated by PCR from genomic DNA (probes A–D) or plasmid pS7C1 (probe E) as indicated in Figure 2. The sequences of oligonucleotides used for PCR are: no. 1 (*easSal*IF), 5′-GACGGAAAAGTTGTGAAGCGTCCGG-3′; no. 2 (*easEco*RIR), 5′-TGACTCCAAATGGAGACGGACCAG-3′; no. 3 (79F), 5′-TTTAAACGCGTCCCCACAA-3′; no. 4 (619F), 5′-CGGAATTCACCTGACATCGCAAATCA-3′; no. 5 (253F), 5′-ATCCATTACCAGTCTGTCAGT-3′; no. 6 (646F), 5′-GCCCGAACCCGTTATGTTCAAC-3′; no. 7 (95F), 5′-ACGGTAGCGGACTGCCAG-3′; no. 8 (620R), 5′-CGGGATCCTTCTTGTGGGGACGCGTT-3′; no. 9 (252R), 5′CGCGATGATTTGCGATGTCAG-3′; no. 10 (254R), 5′-GCAAGGAATACCTCCTGAGTT-3′; no. 11 (78R), 5′-CACGACCAACGTTGTTAAC-3′.

RESULTS

Origin and characteristics of "transparent" isolates: The *cya-8* mutation was first identified in this study. Stable transparent cultures were sent for characterization to Helmut Bertrand, who found them to be deficient in cytochrome aa_3 (personal communication), resembling previously known *cya* mutants (chromosomal; Bertrand *et al.* 1977) and the [*mi-3*] mutant (mitochondrial; Kennell *et al.* 2004). Early observations led to the following hypotheses (Selker 1990): The transparent phenotype obtained in crosses with eas^{UCLA} is due to RIP, which is induced by a duplicate copy of $cya\text{-}8^{+}$ in the eas^{UCLA} parent. The $cya\text{-}8^{+}$ gene was transposed from linkage group (LG) VII and inserted ectopically at or near the *eas* locus in LG II at the time of the original UCLA191 mutation. Crossing the mutant by wild type replaced the *cya-8*-deficient donor

TABLE 1

Incidence of *cya-8* progeny from crosses that are heterozygous or homozygous for different *eas* alleles

Parents[a]	Progeny		
	Nontransparent	Transparent	% transparent
Allele UCLA191			
$eas^{UCLA} \times eas^{+}$ (13)	485	123	20
$eas^{UCLA} \times eas^{UCLA}$ (6)	328	92	22
Allele KH5-9[b]			
$eas^{KH} \times eas^{+}$ (2)	69	0	0
$eas^{KH} \times eas^{KH}$ (2)	110	0	0
Allele JD105			
$eas^{JD} \times eas^{+}$ (5)	386	0	0
$eas^{JD} \times eas^{JD}$	131	0	0
Intercrosses			
$eas^{JD} \times eas^{UCLA}$	38	3	7
$eas^{KH} \times eas^{UCLA}$	68	9	12
$eas^{KH} \times eas^{JD}$	89	0	0

[a] When the numbers were pooled with progeny from more than one cross, the number of crosses is given in parentheses.

[b] Allele KH5-9 (eas^{KH}) is not separable from the translocation *T(IL;IIR)KH5-9 eas*.

chromosome with a normal LG VII, resulting in eas^{UCLA}, a progeny strain that contained two copies of $cya\text{-}8^{+}$.

Transparent *cya-8* mutant progeny are produced whenever eas^{UCLA} is present in one or both of the parents of a cross. In heterozygous crosses, frequencies of transparent progeny are similar regardless of whether eas^{UCLA} was present in the female (protoperithecial) parent or in the male. The mutant progeny show slow, thin growth, producing only a sparse network of hyphae on agar slants 2 days after ascospores are heat-shocked. By the time normal siblings have grown up and conidiated, growth of transparent germinants is still so thin that it usually cannot be seen with the naked eye. Transparents can be readily be distinguished from nongerminants, however, when cultures are examined using a dissecting microscope with transmitted light. Growth of transparent strains is somewhat faster and more vigorous on minimal medium (Vogel 1964; Davis and de Serres 1970) than on glycerol complete medium (Tatum *et al.* 1950). Growth is not improved by supplementing the medium with a variety of growth factors or carbon sources. Different transparent isolates vary in the degree of impairment. After several days, many have grown enough to cover the slant with a thin film that is visible to the naked eye. A few especially vigorous transparent progeny eventually produce sparse conidia, but these strains never attain wild-type growth. At the other extreme, growth of some transparent germinants is so sparse and limited that they fail to cover the agar surface before slants have dried down.

A few germinants that were originally classed as transparent revert to wild type, attaining full growth and conidiation. Conidial pigmentation in such cultures was sometimes yellow rather than orange in the original slant, but in subsequent transfers the conidia were orange. The transferred revertant strains were indistinguishable from their normal siblings, in both phenotype and genotype. During most of this study, strains of this type, *i.e.*, with transitory slow growth, were classed as nontransparent and were pooled with other nontransparents rather than recorded as a distinct class. However, a few of the late-escaping unstable strains may have been misclassified as transparent in the early experiments. Only after the mutant *rid* gene, a suppressor of RIP, came into use was a careful record kept of the transient transparents, which are called "laggards" (Table 6).

Mutation frequency in crosses of different parentage: The frequencies of transparent progeny do not differ greatly in crosses that are heterozygous or homozygous for eas^{UCLA}, or when eas^{UCLA} is used as the male or the female parent. Thirteen crosses in which only one parent was eas^{UCLA} produced 123 transparent progeny among a total of 608 (20%). Six crosses where the parents were both eas^{UCLA} produced 92 transparents among 420 progeny (22%) (Table 1). These are minimum values because some transparent progeny stop growing or grow so slowly that they may be scored as nongerminants, especially if examination is with the unaided eye rather than with the microscope. We have never found a strain containing allele UCLA191 that failed to produce transparent progeny in significant numbers. This was true for crosses parented by >25 different *eas* descendants of the standard eas^{UCLA} strain during 10 generations of outcrossing and backcrossing to the Oak Ridge wild type.

Transparent *cya-8* mutations are not produced by *eas* alleles other than UCLA191: Hasanuma (1984) described an *easily wettable* mutant of independent origin that is inseparable from a reciprocal translocation,

TABLE 2

Location of *cya-8* in linkage group VIIL

Zygote genotype and recombination %	Parentals	Crossovers		
		Singles		
		Region 1	Region 2	Doubles
+ *T(ALS179)*	14	0		
cya-8 *N*	6	1		
5	Plus 13 Barren, all *cya-8*$^+$			
+ *adh* *nic-3*	287	56	45	2
cya-8 + +	—	—	—	—
15 12	Nine crosses pooled			
+ *cyt-7*	—	4		
cya-8 +	—	—		
7	Plus 109 *cya-8*, or *cyt-7*, or *cya-8 cyt-7*			
N *cyt-7*	6[a]	—		
T(ALS179) +	—	0		
0	Plus 11 *cyt-7* Barren[a]			
+ *adh* *nic-3*	19	2	0	0
cyt-7 + +	28	2	1	0
9 4				

Regions are numbered from left to right. In the columns, each pair of classes represents progeny of the two complementary classes. The top number is for the class having the allele of the leftmost locus that is uppermost in the zygote genotype diagram. For example, in the first cross, there were 14 *cya-8*$^+$ *T* progeny, 6 *cya-8 N*, 0 *cya-8*$^+$ *N*, and 1 *cya-8 T*. See Perkins *et al.* (2001) for information on markers. The *cya-8* locus is included in the segment translocated to VR in translocation *ALS179*, but *cyt-7* and other VIIL markers are not included (Perkins 1997). *adh*, adherent morphology; *cya-8*, cytochrome *a* defective; *cyt-7*, cytochromes *a* and *b* defective; *nic-3*, nicotinamide requirement; *N*, normal chromosome sequence; *T*, quasi-terminal translocation *T(VIIL→VR)ALS179*.

[a] The ratio of phenotypically cyt$^+$:cyt$^-$ was 21:44, as expected if the *cyt-7* locus is not included in the translocated segment, which is duplicated in one-third of viable meiotic products. Only 17 of the 44 cyt$^-$ progeny were scored for Barren, *T*, and *N*. Of these, 11 were Barren duplications and 6 were fertile normal sequence, confirming that *cyt-7* is right of the *T(ALS179)* breakpoint in VIIL. The order is thus *cya-8 T cyt-7 adh nic-3*.

T(IL;IIR)KH5-9. He mapped *eas* and the IIR breakpoint near *ace-1* in crosses heterozygous for the translocation and used crosses homozygous for the translocation to show that *eas* is located between *un-20* and *un-15*. For convenience, we refer to *T(IL;IIR)KH5-9 eas* as *eas*^{KH}.

eas^{KH} is allelic with *eas*^{UCLA}. Forced heterokaryons between the two *eas* strains were easily wettable, as were all progeny from *eas*^{KH} × *eas*^{UCLA}. Conidia did not shake loose in tap tests when ascospores from the intercrosses were heat-shocked *en masse* and grown to maturity on plates, or when 63 random isolates were grown to maturity on slants.

Another *eas* allele, *eas*^{JD}, was generated independently by RIP (Bell-Pedersen *et al.* 1992). Unlike *eas*^{UCLA}, the mutant alleles *eas*^{KH} and *eas*^{JD} do not act as mutators (Table 1). Transparent progeny are produced only when *eas*^{UCLA} is present in one or both parents.

Genetic basis of the transparent phenotype: The transparent phenotype is due to a Mendelian mutation that maps in LG VII, 15 map units (MU) distal from what was previously the leftmost gene marker (Table 2). The new locus, named *cya-8* (*cytochrome a-8*), is also left of the breakpoint of the quasi-terminal translocation *T(VIIL → IVR)ALS179*, in which a distal segment of VIIL is translocated to the tip of IVR (Perkins 1997). Progeny from translocation *ALS179* × *Normal sequence* include a viable class that is duplicated for the VIIL segment. Duplication progeny from translocation *ALS179* × *cya-8* are heterozygous for *cya-8* and are phenotypically nontransparent. *cya-8* is phenotypically unlike *cyt-7*, the only other identified cytochrome mutant in VIIL, and 4% wild-type progeny were obtained from intercrossing *cyt-7* × *cya-8*. Sequence of these elements on the genetic map is *Tel-VIIL cya-8 T(ALS179) cyt-7 adh nic-3 . . . Cen-VII*.

eas^{UCLA} is unlinked to VIIL, on the basis of 15 parental ditype (PD):14 nonparental ditype (NPD):34 tetratype (T) asci from *eas*^{UCLA} × *nic-3*, and 25 recombinants between *eas*^{UCLA} and *cya-8* among 48 *cya-8*$^+$ progeny from *nic-3* × *eas*^{UCLA}*; cya-8*. All eight previously described *cya* mutants are located in linkage groups other than VII.

cya-8 is recessive both in partial diploids and in the heterokaryons (*cya-8 A* + *a*^{m1} *ad-3B cyh-1*) and (*cya-8 a* + *a*^{m1}

ad-3B cyh-1), which are phenotypically wild type. Crosses heterozygous for *cya-8* are fully fertile, but perithecia are barren, unbeaked, and completely devoid of ascospores in crosses homozygous for *cya-8*. Expression of the *cya-8* phenotype does not depend on the presence of eas^{UCLA}. The two genes show independent segregation in crosses. Progeny tests show that both eas^{UCLA} and eas^{+} alleles are present among the *cya-8* progeny from intercrosses heterozygous for eas^{UCLA}.

Are independently arising transparents all *cya-8*? Conceivably, the new transparent progeny that originate from nontransparent *eas* parents in different crosses could have resulted from mutation at loci other than *cya-8*. To examine this possibility, transparent progeny were obtained from a series of nine crosses, each of which was parented by a different $cya\text{-}8^{+}$; eas^{UCLA} strain. The eas^{UCLA} strains used as parents had been derived independently, mostly from a series of 10 recurrent backcrosses of eas^{UCLA} to the standard Oak Ridge wild types. Because transparent strains grow too poorly to form protoperithecia and serve as female parents, each putative *cya-8* strain was combined into a heterokaryon with the a^{m1} *ad-3B cyh-1* helper and crossed with a similar *cya-8* heterokaryon of opposite mating type to test for fertility. A cross was also made with *adh nic-3* to test for linkage in VIIL. All nine independently arisen transparent strains proved to be c*ya-8* recurrences by both criteria—barrenness of perithecia in the cross with *cya-8* and linkage left of *adh* in the three-point cross to VIIL markers (Table 3). We conclude that stable eas^{UCLA} -induced transparent mutations are typically *cya-8* and that the mutator activity of eas^{UCLA} is locus specific. It has been assumed without further testing that the stable transparent progeny from subsequent eas^{UCLA}-parented crosses were *cya-8*.

The results described above were obtained with strains derived predominantly from the Oak Ridge wild types. Mutability of the $cya\text{-}8^{+}$ gene under influence of eas^{UCLA} is not limited to strains in the Oak Ridge background, however. Wild-type $cya\text{-}8^{+}$ alleles from five exotic *N. crassa* strains were combined with eas^{UCLA}. Strains from Texas (Mauriceville, P538), India (Aarey, P680), Panama (FGSC 8057), Louisiana (Welsh, P507), and Florida (Groveland, P438) were crossed by a transparent strain containing both *cya-8* and eas^{UCLA}. Fast-growing eas^{UCLA} progeny, which contained the $cya\text{-}8^{+}$ allele from the exotic parent, were then backcrossed to the exotic parent. Transparent progeny obtained from each of these crosses were tested for allelism with *cya-8* by crossing each of them with a standard *cya-8* strain of appropriate mating type, maintained as a phenotypically normal heterokaryon with the a^{m1} *ad-3B cyh-1* helper. Perithecia were barren in each of nine such testcrosses, as expected if the crosses were homozygous for *cya-8* (data not shown). We conclude that wild-type $cya\text{-}8^{+}$ genes of diverse origin are vulnerable to mutation by eas^{UCLA}.

TABLE 3

Evidence that transparent mutations of independent origin map to the *cya-8* region left of *adh* in linkage group VII from crosses of *adh nic-3* (nontransparent) × adh^{+} $nic\text{-}3^{+}$ (transparent)

		Crossovers		
		Singles[b]		
Cross no.[a]	Parentals	*cya-8-adh*	*adh-nic-3*	Doubles
986[b]	43	18	8	1
1184	26	4	1	0
1190	27	12	3	1
1273	51	5	11	0
1335	36	2	6	0
1347	19	5	9	0
1353	35	3	1	0
1357	31	3	6	0
1360	19	4	0	0
Totals	287	56	45	2

[a] Each of the nine crosses was *adh nic-3* × a phenotypically wild-type heterokaryon carrying nuclei of the transparent parent in combination with those of the inactive mating-type *helper-1* strain a^{m1} *ad-3B cyh-1*. Scoring of *adh* and *nic-3* was done only among the nontransparent progeny. Each of the nine transparent strains used in these crosses originated independently from a separate cross that involved a different eas^{UCLA} parent of single-ascospore origin. The eas^{UCLA} parents were from seven different crosses. Allelism of the newly arisen transparent mutations to *cya-8* was also indicated by barrenness when they were crossed with *cya-8* (P9178). Recombination frequencies from the pooled data are *cya-8* 15% *adh* 12% *nic-3*.

[b] Phenotypically transparent progeny were known to be *cya-8* in cross 986 and were inferred from their map position to be at the same locus in the other crossses. The transparent mutation in cross 986 was isolation no. P9178 (FGSC 4523), which was designated *cya-8* and was used for the original mapping.

Mutations to *cya-8* originate in perithecia following fertilization: Experiments using heterokaryons of *cya-8* with the a^{m1} *ad-3B cyh-1* helper showed that homokaryotic *cya-8* conidia give rise to small colonies that are recognizable as transparent under appropriate lighting and magnification. Conidia from an eas^{UCLA} culture that was known to generate transparent sexual progeny in frequencies of 20% or more were plated on sorbose medium. No transparents were found among 145 colonies.

If new *cya-8* mutations were occurring in eas^{UCLA} strains during the vegetative phase, wide fluctuations might be expected in the frequency of transparent progeny from cross to cross, reflecting jackpots due to early mutations in a parent culture. Frequencies might also be related to the age and history of the eas^{UCLA} cultures used as parents. The fact that observed frequencies of transparent progeny are rather uniform regardless of the age of the eas^{UCLA} parents, combined with the failure to demonstrate the presence of transparent nuclei in vegetative cultures of eas^{UCLA}, suggested that mutation

occurs only at fixed times during the sexual part of the life cycle. Experiments were therefore designed that would set limits to the period during which *cya-8*$^+$ is subject to mutation.

All asci of an individual perithecium usually trace their origin to the single pair of haploid nuclei of opposite mating type that came together in the archegonium at the time of fertilization (Nakamura and Egashira 1961; Johnson 1976). If mutation occurred in one of the nuclei at the time of fertilization or before, 50% of ascospores in each affected perithecium would be mutant and all asci in the perithecium would contain four *cya-8* and four *cya-8*$^+$ ascospores. If, on the other hand, mutation occurred at some later time during perithecial development, then <50% of ascospores would be mutant. Only some of the asci in an affected perithecium would contain both normal and mutant ascospores, while in the remaining asci, eight ascospores would be normal *cya-8*$^+$. The number of asci in which mutant ascospores are present, and the number of mutant ascospores per ascus, would depend on the stage of development when mutation occurred.

The wild-type strain ORS-6a was crossed with *eas*^{UCLA}. Ten days after fertilization, individual perithecia that had not yet expelled ascospores from the ostiole were selected. The contents of individual perithecia were squeezed out one by one into separate water drops in a sterile petri dish and asci were broken up so as to release ascospores. After aging for 10 days at 30°, the ascospores from each water drop were transferred by Pasteur pipette or loop to sorbose–minimal 3% agar medium in a petri dish. The ascospores were spread and heat-shocked 30 min at 60°, incubated at 34°, and examined 24 and 48 hr later to determine the numbers of transparent and nontransparent progeny. Transparent progeny were produced by each of the eight perithecia sampled in frequencies that ranged from 9 to 16% (12% overall for 785 colonies). It appears that transparent progeny arise from events in the perithecia subsequent to fertilization. The stage at which mutation occurred was similar in different perithecia, and jackpots were not detected.

***cya-8* mutations occur prior to karyogamy:** The experiment just described sets an early limit to the time of mutation. The distribution of *cya-8* mutations in individual asci enables a later limit to be defined. Mutations that occur prior to the premeiotic S-phase and karyogamy would be expected to result in asci showing 4:4 segregation for *cya-8*. If mutation occurred later than premeiotic DNA synthesis, then fewer than four of the eight ascospores in individual asci should produce transparent progeny.

Unordered asci from the cross *eas*^{UCLA} × *adh nic-3* were obtained as groups of eight ejected ascospores, using the procedure described by Strickland (1960) and Perkins (1966). The ascospores were aged, transferred individually to slants in 10 × 75-mm tubes, heat-shocked, incubated 3 days at 34°, and examined for the presence of *cya-8*. Among 83 asci with seven or eight ascospores germinated, *cya-8* was present in 11. Each of these showed 4:4, 4:3, or 3:4 segregation for *cya-8*:*cya-8*$^+$. Mutant *cya-8* progeny were also obtained from asci with only five or six ascospores germinated. The content of each of these incomplete asci was also consistent with 4:4 segregation. The overall frequency of asci that had acquired a mutant *cya-8* allele was 15% (14/95).

We conclude that *cya-8* mutations occur in ascogenous hyphae prior to the premeiotic S-phase, which immediately precedes karyogamy. The ascogeneous hyphae are two-component heterokaryons populated with haploid *A* and *a* nuclei that are derived from a single original pair following fertilization. Similar criteria were used by Selker *et al.* (1987) to establish that RIP occurs between fertilization and karyogamy in nuclei containing a duplication and by Butler and Metzenberg (1989) to determine the timing of premeiotic changes in the number of rDNA repeats in the nucleolus organizer.

Mutation occurs preferentially or exclusively in the nucleus that contains *eas*^{UCLA}: The asci containing newly arisen mutant *cya-8* alleles in the experiment just described originated from a cross in which markers linked to *cya-8* were segregating. If induction of mutations was equally probable in the two nuclear components of the heterokaryon, *eas*^{UCLA} *a* and *adh nic-3 A*, then linkage of *cya-8* to the nearby markers would be obscured. Results clearly indicated the contrary (Table 4A). The new *cya-8* mutations were closely linked in *cis* to the *nic-3* allele that was present in LG VII in the *eas*^{UCLA} parent. The progenitor nuclei of these asci must have been *eas*^{UCLA}*; cya-8 adh*$^+$ *nic-3*$^+$ and *eas*$^+$*; cya-8*$^+$ *adh nic-3*. The *cya-8* and *nic-3* markers segregated so as to give 10 parental ditypes, 0 nonparental ditypes, and 3 tetratypes (12% recombination), as expected for this coupling phase. Induction of *cya-8* mutations is therefore restricted to nuclei that contain the mutant *eas*^{UCLA} allele.

Further evidence that mutation occurs preferentially in the *eas*^{UCLA} nuclei was obtained using random ascospore isolates from crosses heterozygous for VIIL markers (Table 4B). The newly arisen *cya-8* alleles consistently showed linkage in *cis* phase to the marker allele that was present in the *eas*^{UCLA} nucleus.

Mutation of *cya-8* in *eas*^{UCLA} strains is suppressed by large segmental duplications: Bhat and Kasbekar (2001) have shown that when both a large duplication and a small duplication are present in a cross, RIP is suppressed in the small duplication, suggesting that the two duplications compete for access to the RIP machinery. Suppression is stronger when the large duplication is homozygous than when it is heterozygous (Fehmer *et al.* 2001). The hypothesis that *cya-8* mutations are due to RIP was tested by crossing *eas*^{UCLA} with two strains that contain long segmental duplications. Although crosses

TABLE 4

Evidence from random ascospores and from tetrads that new *cya-8* occurrences originate preferentially or exclusively in the nucleus that contains *eas*^{UCLA}

Parental genotypes (all *cya*$^+$)	Unordered asci that contain four *cya-8* progeny				No.	Ascus type[a]	% *cya-8–nic-3* recombination
	cya nic	*cya nic*	*cya nic*	*cya nic*			
				A. Asci			
eas^{UCLA}*; nic-3*$^+$ × *eas*$^+$*; nic-3*	− (+)	− (+)	+ −	+ −	10	PD	12
	− (+)	− (−)	+ +	+ −	3	T	
	− (−)	− (−)	+ +	+ +	0	NPD	
eas^{UCLA}*; nic-3* × *eas*$^+$*; nic-3*$^+$	− (−)	− (−)	+ +	+ −	32	PD	25
	− (+)	− (−)	+ +	+ −	21	T	
	− (+)	− (+)	+ −	+ −	1	NPD	

Parent genotypes (all *cya*$^+$)	No. and genotype of *cya-8* progeny[b]	% *cya-8–nic-3* recombination[c]
	B. Random isolates	
eas^{UCLA}*; nic-3*$^+$ × *eas*$^+$*; nic-3*	41 *nic*$^+$, 13 *nic-3*	25
eas^{UCLA}*; nic-3* × *eas*$^+$*; nic-3*$^+$	8 *nic*$^+$, 35 *nic-3*	19
Pooled		22

The genotype of each ascospore pair is given. The *nic-3* marker was not scored in the slow-growing cytochrome-deficient progeny. However, the inferred allele is shown in parentheses. The percentage of asci in which a new *cya* mutation was segregating was 15% in the first cross and 55% (54/98) in the second. *eas* and *cya-8* were unlinked (3 PD, 46 T, and 5 NPD in the second cross). PD, parental ditype; T, tetratype; NPD, nonparental ditype.

[a] PD and NPD would be equally frequent if mutation to *cya-8* were equally likely to occur in *eas*^{UCLA} *and eas*$^+$ nuclei. The linkage observed (PD > NPD) would be expected if the mutant *cya-8* allele originated in the *eas* nucleus.

[b] *cya-8* progeny were rescued by co-inoculating with *nic-3 arg-10 A* or *nic-3 arg-10 a* on slants containing nicotinamide but not arginine. The resulting vigorous heterokaryons were then tested on minimal medium to determine whether the *cya-8* component was *nic-3* or *nic-3*$^+$.

[c] Assuming that *cya-8* originated in the *eas*^{UCLA} nucleus. Recombination between *cya-8* and *nic-3* is 26% in the conventional crosses in Table 1.

are barren when these duplications are present, they are not completely unproductive and a few ascospores are produced. The frequency of transparent progeny is drastically reduced in crosses with *eas*^{UCLA} that are heterozygous or homozygous for either of the large duplications (Table 5). This is as would be expected if RIP is responsible for the production of *cya-8* mutations by *eas*^{UCLA}.

Mutation of *cya-8* in *eas*^{UCLA} strains is suppressed in the presence of a mutation that abolishes RIP: Discovery of the recessive RIP-defective gene *rid* (Freitag *et al.*

TABLE 5

Effect of long duplications on the production of *cya-8* progeny in crosses with *eas*^{UCLA} heterozygous or homozygous

Parents[a]	Progeny		
	Nontransparent	Transparent	% transparent[b]
eas^{UCLA} × *eas*$^+$*; Dp(OY329)*	128	1	1
eas^{UCLA}*; Dp(OY329)* × *eas*^{UCLA}*; Dp(OY329)* (3)	211	0	0
eas^{UCLA} × *eas*$^+$*; Dp(S1229)*	115	1	1
eas^{UCLA}*; Dp(S1229)* × *eas*^{UCLA}*; Dp(S1229)* (4)	157	0	0

[a] When the numbers were pooled for progeny from more than one cross, the number of crosses is given in parentheses. *eas*^{UCLA}*; Dp(OY329)* was obtained as a duplication progeny from *T(VIR→ IIIR)OY329)* × *eas*^{UCLA}, and *Dp(S1229)* was obtained as a duplication progeny from *T(IV→ VIIL; IL;IIL; IVR)S1229* × *eas*^{UCLA}; see Perkins (1997).

[b] In the absence of *Dp(OY329)* or *Dp(S1229)*, these crosses would be expected to produce ~20% transparent progeny (see Table 1). Bhat and Kasbekar (2001) and Fehmer *et al.* (2001) suggest that these long duplications compete effectively for the RIP machinery, decreasing the frequency with which a small duplication in the same nucleus undergoes RIP.

TABLE 6

Effect of the RIP-defective gene *rid* on the frequency of stable *cya-8* mutations among progeny of crosses involving *eas*^{UCLA}

Parents[a]	Progeny: Stable transparents[b]	Laggards[c]	Total
rid homozygous			
rid; eas^{UCLA} × *rid; eas*^{UCLA} (2)	2	13	300
rid; eas^{UCLA} × *rid*	6	5	212
rid; eas^{UCLA} × *rid; sad-2*	1	2	266
Total	9 (1)	17 (2)	778
rid heterozygous			
rid; eas^{UCLA} × *eas*^{UCLA}	10	11	154
rid; eas^{UCLA} × WT (2)	18	5	143
rid; eas^{UCLA} × *sad-2*	15	0	92
Total	43 (11)	16 (4)	389
No *rid*[d]			
eas^{UCLA} × *eas*^{UCLA}	16	0	94
eas^{UCLA} × WT	15	0	114
Total	31 (15)	0	208
No *eas*^{UCLA}			
rid × *rid* (2)	1	6	214
WT × WT	0	1	253
Total	1 (<1)	7 (1)	467

Numbers in parentheses are percentages.

[a] When the numbers were pooled for progeny from more than one cross, the number of crosses is given in parentheses. WT (wild type): OR23-1VA or ORS-6a. *rid* (*RIP-deficient*): suppressor of RIP. *sad-2* (*suppressor of ascus dominance*): suppressor of MSUD.

[b] Stable transparents are expected to result from RIP of duplicated *cya-8*$^{+}$.

[c] Laggards are defined as progeny that were classified as transparent 3 days after germination, but that reverted to normal subsequently.

[d] The 19 crosses of *eas*^{UCLA} × *eas*^{UCLA} .or *eas*^{UCLA} × *eas*$^{+}$ in Table 1, with 21% transparent progeny, might also be taken as "No *rid*" controls. The two additional crosses reported here were made because laggards were not recorded as such when the numbers were obtained for Table 1. At that time, most laggards would have been scored as nontransparent, but a few laggards may have been classified as transparent.

2002) provided a good opportunity to test the hypothesis that the *cya-8* mutations in transparent progeny are caused by RIP. If RIP is responsible, then crosses homozygous for *rid* and homo- or heterozygous for *eas*^{UCLA} should show reduced numbers of transparent progeny, or none at all. In accordance with this expectation, the frequency of transparent progeny was reduced in crosses homozygous for *rid* (Table 6). However, some progeny that showed the slow, transparent growth typical of *cya-8* mutations were still produced. Most of these were laggards, reverting to wild type during vegetative growth, whereas most transparent progeny from control crosses were stable. Recognition that transparent progeny are of two types, stable and unstable, suggested that the unstable type was caused by something other than RIP, which introduces C:G to T:A changes that are highly stable. It seems unlikely that the unstable transparents were due to quelling, a silencing process that silences the expression of duplicated genes reversibly during the vegetative phase. As shown above, mutations to *cya-8* were not detected during vegetative growth of *eas*^{UCLA}.

Mutation of *cya-8* in *eas*^{UCLA} strains is not suppressed by a mutation that suppresses meiotic silencing by unpaired DNA: Meiotic silencing by unpaired DNA (MSUD) results in epigenetic inactivation of genes that are unpaired in meiotic prophase (Aramayo and Metzenberg 1996, Shiu *et al.* 2001; Shiu and Metzenberg 2002). Silencing is temporary: MSUD is not known to produce stable mutations. We used a suppressor of MSUD called *Sad-2* (*Suppressor of ascus dominance*) (Shiu *et al.* 2006) to show that MSUD is not involved in the production of stable transparent progeny (Table 6).

Although observations of silencing by MSUD are usually limited to genes that are expressed during the sexual phase, the possibility remains that, for some genes, function may not be restored until after ascospore germination. Further experiments will be needed to determine whether MSUD is responsible for the occasional transiently inhibited "laggard" progeny that have been classified as transparent but that are subsequently seen to revert to normal growth.

Effectiveness of *eas*^{UCLA} as a mutator after repeated exposure to RIP: Cambareri *et al.* (1991) showed that the frequency of RIP decreased after duplicated segments had passed through successive crosses that subjected them repeatedly to alteration by RIP. After many generations, RIP-induced divergence was lower for unlinked duplications than for linked duplications To see whether the mutator effect of *eas*^{UCLA} declined in similar fashion, numbers of *cya-8* progeny were determined after *eas*^{UCLA} had been exposed repeatedly to RIP. The *eas*^{UCLA} allele was tagged with *fl*^{DE1}, with which it does not recombine. *eas*^{UCLA} *fl*^{DE1} was crossed with *fl*$^{+}$ *trp-3* to obtain a prototrophic transparent F_1 progeny of constitution *eas*^{UCLA} *fl*^{DE1}*; cya-8*. This, in turn, was crossed with *fl*$^{+}$ *trp-3* to obtain nontransparent *eas*^{UCLA} *fl*^{DE1} backcross progeny. These were designated *eas*$^{RIP\ 1}$ *fl* to signify that the *eas* allele had been altered by one round of exposure to RIP. The cycle was repeated eight times to obtain *eas*$^{RIP\ 8}$ *fl*. When this eight-times exposed strain was crossed by wild type, 14 of 123 progeny (11%) were stably transparent, a frequency unchanged from the 14/129 (11%) transparents obtained among progeny of the parental, unexposed *eas*$^{RIP\ 0}$ *fl* × wild type. Thus, mutator activity of *eas*^{UCLA} appears not to have been impaired by repeated exposure to RIP.

Cytogenetic complexity of the *eas*^{UCLA} mutation: The point-mutant allele *eas*^{JD} was used to determine standard wild-type gene order in LG IIR. In a cross of *un-20 eas*^{JD} ×

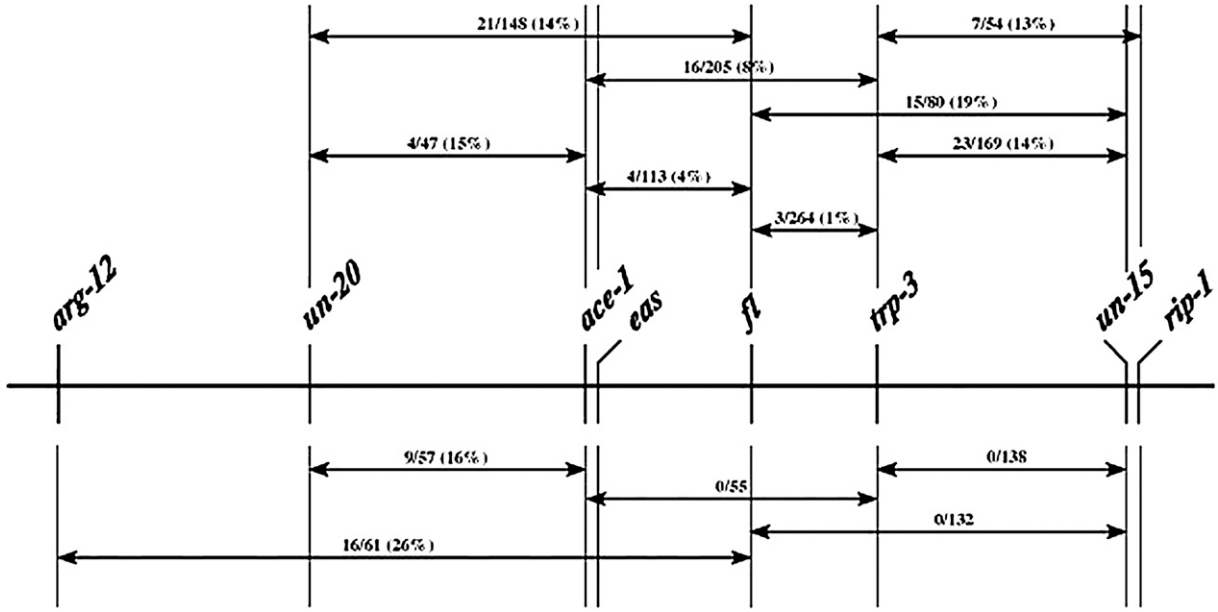

FIGURE 1.—Crossing-over frequencies between IIR markers in crosses of $eas^{+} \times eas^{+}$ (above the map) and $eas^{+} \times eas^{UCLA}$ (below the map), based on data in Tables 7 and 8. When eas^{UCLA} is homozygous, the order of *fl* and *trp-3* is reversed and recombination values are *ace-1* (16%) and *trp-3* (11%) *fl*, with no double crossovers among 73 progeny, and *un-20* (16%) and *trp-3* (12%) *fl*, with no double crossovers among 76 progeny (Table 8). Markers: *arg-12*, *ace-1*, *trp-3* (auxotrophs); *fl*: *fluffy* (morphological); *un*: unknown function (temperature sensitive conditional); *rip-1*: ribosome production (temperature sensitive conditional).

ace-1, 3 progeny among 290 were recombined for *ace-1* and *eas*. Two of these crossovers were *un-20 ace-1*$^{+}$ *eas*$^{+}$ and one was *un-20*$^{+}$ *ace-1 eas*. Gene order is therefore *un-20 ace-1 eas*, with the *eas* locus right of *ace-1* (Figure 1).

When eas^{UCLA} is heterozygous, recombination between IIR markers right of *ace-1* is blocked in a region that is normally 25 MU long (Figure 1; Table 7). Crossing over left of *ace-1* remains normal. These results suggest that a distal segment of IIR with one breakpoint at or near *eas* became inverted at the same time that the UCLA191 mutation occurred at the *eas* locus. Simultaneously, a copy of *cya-8* might then have been inserted at one of the inversion breakpoints.

Direct evidence of inversion would be provided by crosses that are homozygous for the rearrangement and heterozygous for at least three markers—two inside the putative inverted segment and one outside. Strains were available in which eas^{UCLA} was recombined with the outside markers *ace-1* and *un-20*, which are left of both *eas* and the recombination block. No recombinants were obtained when eas^{UCLA} was crossed with the rightmost linkage group II markers *un-15* or *rip-1*, which may or may not be included in the inversion. We succeeded in inserting the included marker *trp-3* by recombination. The *fl* (*fluffy*) gene is positioned to provide a second included marker. Because *fluffy* strains are aconidiate and *fl eas* cannot be distinguished phenotypically from *fl* *eas*$^{+}$, a mutant *fl* allele could not be introduced by recombination. Instead, *fluffy* was introduced into eas^{UCLA} by RIP. The wild-type *fl*$^{+}$ *gene* had been cloned and sequenced by BAILEY and EBBOLE (1998). Transformation was carried out using a plasmid that contained both *fl*$^{+}$ and a selectable marker conferring hygromycin resistance, as described in MATERIALS AND METHODS. A purified hygromycin-resistant transformant of eas^{UCLA} was crossed with an eas^{UCLA} strain of opposite mating type. Because transformation was by ectopic integration, the transformed parent carried two copies of *fl*$^{+}$. Consequently, its progeny included RIP-induced *fluffy* mutations. A hygromycin-sensitive eas^{UCLA} *fluffy* strain from among the progeny was crossed to *un-20* eas^{UCLA} *trp-3* and to *ace-1* eas^{UCLA} *trp-3*. The results (Table 8) show clearly that the order of *fl* and *trp-3* is reversed in eas^{UCLA}. We conclude that the eas^{UCLA} mutation resulted from a complex rearrangement, which might be symbolized as *T(VIIL→IIR) In(IIR)UCLA191 eas*.

Cytological observations of meiosis: Meiotic chromosomes were examined in asci heterozygous for eas^{UCLA}. If the relatively inverted segments were paired homologously, a loop might be seen at pachytene, and crossing over within the inversion would result in the formation of anaphase bridges and fragments. No obvious abnormalities were seen. Pairing appeared to be normal, and bridges and fragments were not apparent in preparations made using aceto-orcein (E. G. BARRY, personal communication) or DAPI (N. B. RAJU, personal communication). BOJKO (1990) has used synaptonemal complex reconstructions to demonstrate that synaptic adjustment occurs in Neurospora when long inversions are heterozygous, resulting in extensive nonhomologous pairing. Thus, it might be expected that small inversions would be paired nonhomologously at late

TABLE 7

Evidence for inversion in crosses heterozygous for *eas*^{UCLA}: suppressed crossing over of IIR markers right of *eas*

Zygote genotype[a] and recombination %	Parentals	Crossovers		
		Singles		
		Region 1	Region 2	Doubles
Homozygous normal sequence				
+ *ace-1*	24	4		
un-20 +	16	3		
15				
eas^{UCLA} heterozygous				
* + +	26	2		
un-20 *ace-1*	22	7		
16				
Homozygous normal sequence				
+ *trp-3*	45	5		
ace-1 +	37	5		
11				
eas^{UCLA} heterozygous				
* + *trp-3*	37	0		
ace-1 +	18	0		
0				
Homozygous normal sequence				
+ *un-15*	48	5		
trp-3 +	30	10		
16				
+ +	29	4		
trp-3 *un-15*	40	4		
10				
eas^{UCLA} heterozygous				
+ *un-15*	25	0[b]		
* *trp-3* +	46	0		
0				
* + +	37	0		
trp-3 *un-15*	30	0		
0				
Homozygous normal sequence				
+ + +	24	14	9	0
pe *fl* *un-15*	18	9	6	0
29 19				
eas^{UCLA} heterozygous				
* + + +	21	16	0	0
pe *fl* *un-15*	22	13	0	0
40 0				

(*continued*)

pachytene and that they would therefore go undetected when bivalents were examined using light microscopy.

Molecular evidence for RIP of the *cya-8* copy that adjoins *eas*^{UCLA}: Molecular comparisons based on restriction fragment length polymorphisms (RFLPs) detected by Southern hybridizations between *eas*^{UCLA} and its progenitor strains (Bell-Pedersen *et al.* 1992; Lauter *et al.* 1992) suggested that the *eas* allele is associated with an insertion of at least 2 kb, probably interrupting promoter elements required for normal transcription levels

TABLE 7

(Continued)

Zygote genotype[a] and recombination %	Parentals	Crossovers		
		Singles		Doubles
		Region 1	Region 2	
Homozygous normal sequence				
+ + +	12	9	2	0
arg-12 *fl* *rip-1*	17	9	5	0
33 13				
eas^{UCLA} heterozygous				
* + + +	22	12	0	0
arg-12 *fl* *rip-1*	23	4	0	0
26 0				

Normal sequence is *pe arg-12 un-20 ace-1 eas fl trp-3 un-15 rip-1*. Conventions as in Table 2. Distance between markers is approximately as follows in the normal sequence IIR map that uses the point-mutant *eas* allele JD105: *pe* (1%, 5%) *arg-12* (16%) *un-20* (15%) *ace-1* (1%) *eas*^{JD} (9%) *fl* (2%, 6%) *trp-3* (10%) *un-15* (1%) *rip-1*.

[a] The *eas* parent is indicated by an asterisk in crosses heterozygous for *eas*^{UCLA}.

[b] One phenotypically Trp$^+$ Un$^+$ progeny proved to be a pseudowild heterokaryon. This produced *trp-3 un-3*$^+$ and *trp-3*$^+$ *un-3* progeny when testcrossed.

(Bell-Pedersen *et al.* 1996). As described above, we suspected that transposed, duplicated *cya-8* sequence is part of this insertion. The molecular identity of the *cya-8* gene remains unknown, even though the Neurospora genome is almost completely sequenced (Galagan *et al.* 2003) and many of the identified Saccharomyces or human genes involved in cytochrome synthesis have homologs in Neurospora. Cloning the *cya-8* gene by complementation was a poor option because *cya-8* strains grow poorly and form few asexual spores, making them difficult to transform. Instead, we attempted to isolate part of the *cya-8* gene integrated at the *eas* locus. Standard long-range PCR with outside primers has failed. Alternative approaches (*e.g.*, inverse PCR from genomic DNA isolated from *eas*^{UCLA} strains or construction of partial plasmid libraries with gel-purified 5.3- and 8-kb *Sau*3AI fragments) were also attempted repeatedly but did not yield the desired breakpoint fragments.

To provide molecular evidence for the hypothesis that the transparent progeny from *eas*^{UCLA} crosses are caused by RIP of *cya-8*, we assayed for DNA methylation in the *eas* region. While point mutations induced by RIP are usually contained within the duplicated segments, DNA methylation frequently spreads outside the duplication and has been found thousands of base pairs from the duplicated region (*e.g.*, see Foss *et al.* 1991; Irelan and Selker 1997; Miao *et al.* 2000). We therefore reasoned that if *cya-8* mutations are caused by RIP, DNA methylation may be present in the *eas*^{UCLA} promoter region. We inspected the *eas* promoter region for telltale RFLP and/or DNA methylation by Southern analysis using the 5-methylcytosine-insensitive *Dpn*II and its 5-methylcytosine-sensitive isoschizomer, *Sau*3AI (Figure 2; data not shown). In *eas*^{UCLA} strains, probes B and C revealed RFLPs when DNA was digested with *Dpn*II. A 395-bp band present in the wild type was replaced by an ~480-bp band. Both *eas*$^+$ and the nonmutator *eas*^{JD} alleles showed the expected 395-bp band as well as a 406- or 441-bp band for probes B and C, respectively. Both the 406- and 441-bp fragments appeared unchanged in the *eas*^{UCLA} strain. This suggests that both the insertion containing *cya-8*$^+$ and the inversion breakpoint are present in the 395-bp interval (shown as a shaded box in Figure 2A). Previous molecular analyses (Bell-Pedersen *et al.* 1992; Lauter *et al.* 1992) suggested that *eas*^{UCLA} contains an insertion that interrupts important promoter elements (Bell-Pedersen *et al.* 1996; Rerngsamran *et al.* 2005). Induction of *eas* during conidiation requires the *fluffy* transcription factor FL. The strongest binding site for FL in the *eas* promoter lies within the 395-bp interval, 129 bp from the 3′-end of the fragment. This binding site is important for *eas* expression *in vivo* (Rerngsamran *et al.* 2005). We predict that one end of the insertion/inversion lies within this 129-bp interval, separating the FL-binding site from *eas*.

Both probe B and probe C detected *Sau*3AI fragments of ~5.3 and ~8 kb (Figure 2B). These high-molecular-weight bands presumably stem from methylation of *Sau*3AI sites contained in the insertion that bears *cya-8*$^+$. The insertion is apparently heavily methylated and is perhaps longer than previously suggested (Lauter *et al.* 1992). We did not detect any methylation in the *eas*^{UCLA} region outside of that corresponding to the wild-type 395-bp band (probes A, D, E; Figure 2). Analyses of strains from earlier *eas*^{UCLA} × wild type crosses revealed bands resulting from RFLPs and incomplete methylation (data not shown), suggesting that the severity of DNA methylation and RIP increased in successive generations.

TABLE 8

Altered sequence of markers in crosses homozygous for *eas*^{UCLA} compared to standard sequence

Zygote genotype and recombination %	Parentals	Crossovers		
		Singles		
		Region 1	Region 2	Doubles
Homozygous normal sequence				
+ *fl* +	107	4	2	0
ace-1 + *trp-3*	—	—	—	—
4 2				
eas^{UCLA} homozygous[a]				
+ + *fl*	53	12	8	0
ace-1 *trp-3* +	—	—	—	—
16 11				
Homozygous normal sequence				
+ *fl* *trp-3*	30	6	3	0
un-20 + +	28	6	0	0
16 4				
+ *fl* +	32	6	2	0
un-20 + *trp-3*	32	3	1	0
12 4				
eas^{UCLA} homozygous				
+ + *fl*	16	15	5	0
un-20 *trp-3* +	29	7	4	0
16 12				
Homozygous normal sequence				
+ *trp-3*	45	5		
ace-1 +	37	5		
11				
eas^{UCLA} homozygous				
+ *trp-3*	15	7		
ace-1 +	19	1		
19				

Conventions are as in Table 2.
[a] The *ace-1 trp-3* parent also carried the point mutant *eas*^{ID101}.

DISCUSSION

The cause of recurrent mutation: The observations reported here support Selker's (1990) hypothesis that existing strains of *eas*^{UCLA} contain two copies of the *cya-8*$^{+}$ gene, one at its original location in LG VIIL and the other linked to *eas* in IIR. This model predicts that mutant *cya-8* progeny will be produced by RIP whenever the duplication-bearing *eas*^{UCLA} strain is present in one or both parents of a cross. The original UCLA191 mutation is visualized as a four-break complex rearrangement in which *cya-8*$^{+}$ was removed from VII and inserted in II at one of the breakpoints of a paracentric inversion that disrupted *eas* (see Figure 3). About one in five translocations in Neurospora is insertional rather than reciprocal, and complex, multiple-break rearrangements are not uncommon (Perkins 1997).

The original UCLA191 mutant strain was not saved. Instead, *eas* progeny of repeated backcrosses to the standard Oak Ridge wild types were retained as stocks (Selitrennikoff 1976). Backcrossing would have replaced the original deleted donor chromosome with a wild-type copy of linkage group VII. As a result, two copies of *cya-8*$^{+}$ are expected to be present in each nucleus in the *eas*^{UCLA} stocks that were retained and that were used in this study.

Predictions for RIP: Mutations originating by RIP are expected to be stable. If the *eas*^{UCLA}-induced stable *cya-8* mutations are in fact caused by RIP, testable predictions can be made:

1. *cya-8* mutations should be abolished or decreased in frequency in crosses homozygous for the recessive RIP-deficient mutation *rid* (Freitag *et al.* 2002).

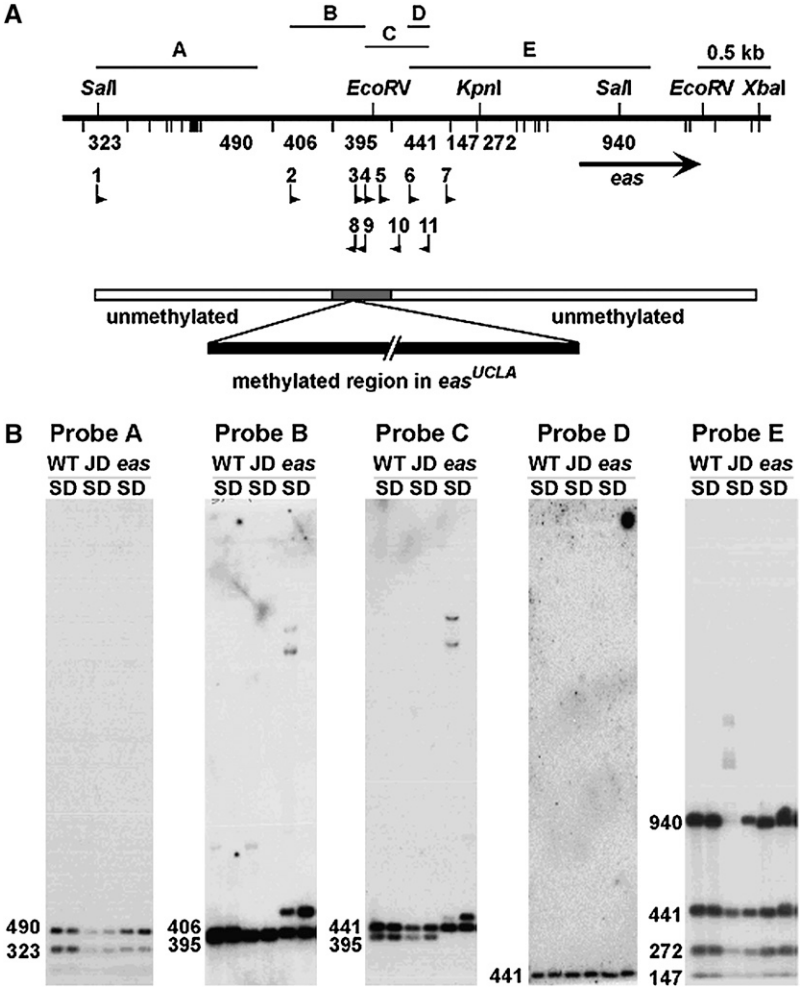

FIGURE 2.—Molecular analysis of the *eas*^{UCLA} allele. (A) Partial map of the *eas*$^{+}$ gene and its transcript (arrow). Selected restriction endonuclease sites that were used in previous studies are indicated (BELL-PEDERSEN *et al.* 1992; LAUTER *et al.* 1992). *Dpn*II/*Sau*3AI sites are shown as vertical marks below the horizontal line, the size of selected fragments expected in the Southern blots in B is noted, and the position of probes A–E used for B is shown. Primers used in this study (see MATERIALS AND METHODS) are shown as inverted flags with arrow points to indicate orientation. The shaded segment shows the region of discontinuity between normal (open) and mutated (solid) DNA in *eas*^{UCLA}. (B) Southern analysis of the proximal *eas*^{UCLA} region. Genomic DNA of wild-type strain 74-OR23-IVA (WT), *eas*^{JD} (JD), and *eas*^{UCLA} (*eas*) was digested with *Dpn*II and *Sau*3AI; *Sau*3AI is sensitive to cytosine methylation. Only probes B and C detect higher-molecular-weight bands in DNA from the *eas*^{UCLA} strain, presumably caused by DNA methylation (note the concomitant disappearance of the ~480-bp band in the *eas Sau*3AI lanes). Wild-type strains show no DNA methylation throughout the region tested. The *eas*^{JD} allele is methylated in the *eas* coding region because this allele was generated by RIP (BELL-PEDERSEN *et al.* 1992). These data suggest that the insertion/inversion occurred in the 395-bp segment shown in A and that this segment likely contains both *cya-8*$^{+}$ insertion breaks and one *eas*^{UCLA} inversion breakpoint. Therefore DNA to the right of the solid box in A is inverted distally toward the telomere in *eas*^{UCLA}.

2. The frequency of stable *cya-8* mutant progeny may be decreased when *eas*^{UCLA} is crossed to a partial-diploid strain that contains a long nonhomologous duplication. This prediction is based on the results of BHAT and KASBEKAR (2001), who suggested that a long duplication out-competes a small duplication for the RIP machinery when both duplications are present in a cross. The competition occurs in dikaryotic ascogenous hyphae prior to meiosis, and it is effective regardless of whether the long and short duplications are in the same nucleus or in separate nuclei.
3. The ability of a duplicated DNA segment to trigger RIP might be expected to decrease as it is passed through successive crosses that subject it to RIP. The repeated exposure would subject it repeatedly to alteration, making it less effectively recognized by the RIP machinery (CAMBAREri *et al.* 1991).

The first two predictions have been confirmed (Tables 5 and 6). However, we saw no reduction in the frequency of stable transparent progeny after eight successive crosses in which *eas*^{UCLA} was subjected to RIP. This is perhaps not surprising, because the duplicated sequences in question are unlinked, and RIP was shown by CAMBAREri *et al.* (1991) to continue through seven generations when an unlinked duplication of an ~6-kb sequence was passed repeatedly through crosses. We did note an increase in the size of methylation fragments from the *eas*^{UCLA} promoter region when comparing progeny from early *vs.* later *eas*^{UCLA} crosses (data not shown). This suggests that RIP continued to occur, resulting in more extensive DNA methylation in successive crosses.

Other possible explanations for the mutator effect: Explanations other than RIP have been considered. Experimental results are inconsistent with origin of the stable, heritable *cya-8* mutations by any of the following: quelling, meiotic silencing of unpaired DNA, mitochondrial mutation, transposition of mobile elements, or deletion at meiosis in heteroallelic repeats.

1. Quelling might be evoked to account for those transparent isolates that are unstable. Quelling, which inactivates duplicated genes during the vegetative phase (ROMANO and MACINO 1992; COGONI *et al.* 1996; COGONI and MACINO 2000), is usually detected in isolates that acquire ectopic insertions, typically with multiple copies of the sequences,

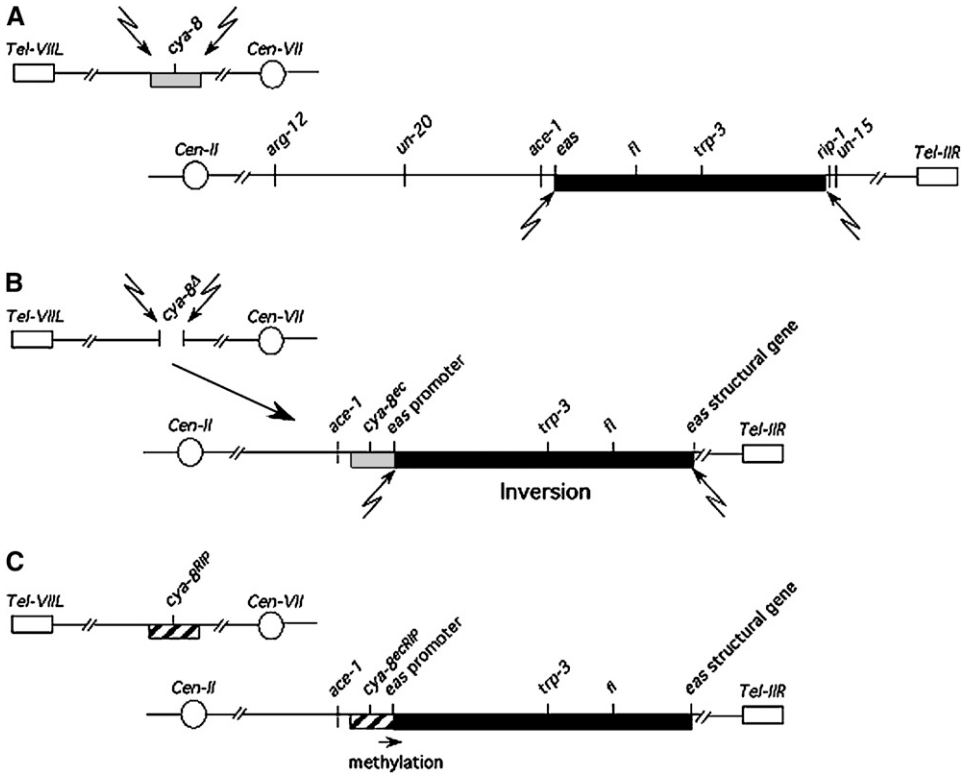

FIGURE 3.—Model for the coupled insertional translocation/inversion event that produced the *eas*^{UCLA} mutation and for the production of *cya-8* mutations by RIP. (A) Partial map of normal-sequence LGs II and VII with putative breakpoints, showing presumed double-strand breaks (lightning bolts), the approximate extent of the inverted segment in LG IIR (solid box), and the deleted segment in LG VIIL (shaded box). Because no crossovers have been obtained between the rearrangement and the *rip-1* or *un-15* loci, it is not known whether these genes are inside or outside the inverted segment. (B) Generation of the *eas*^{UCLA} mutation by a combined insertional translocation and inversion. Chromosome constitution following the coupled translocation/inversion. A fragment of LG VIIL harboring *cya-8* was translocated to LG IIR and inserted centromere proximal of the *eas* promoter, resulting in deletion of *cya-8* from LG VIIL (*cya-8*$^{\Delta}$) and its ectopic insertion into LG IIR (*cya-8*^{ec}). Concomitantly, a segmental inversion of LG IIR inactivated *eas*, moving the coding region closer to the telomere. The exact extent of the translocated fragment and of the inversion is not known, nor do we know if small deletions occurred at sites of the presumed double-strand breaks (lightning bolts). Evidence that the *eas* locus is split is provided by our inability to amplify fragments spanning the *eas* promoter and the 5′ region of *eas*, using *eas*^{UCLA} or strains derived from it. (C) Constitution of a "transparent" backcross progeny that is mutant for *cya-8*. In a backcross of the *eas cya-8* mutant to a wild-type strain, one-half of the mutant *eas* progeny are expected to be duplicated for *cya-8*$^{+}$, having received one copy in an intact LG VII from the wild-type parent and carrying a second copy (*cya-8*^{ec}) in the rearranged LG II from the mutant *eas*^{UCLA} parent. Duplication of the *cya-8* region results in premeiotic C:G to A:T mutations by RIP in both segments. During a backcross of one of these *eas cya-8* duplication stocks, both *cya-8* copies were mutated by RIP (*cya-8*^{RIP} and *cya-8*^{ecRIP}, crosshatched boxes), producing the chromosomes diagrammed here. DNA methylation is usually, but not always, found in segments mutated by RIP (for review see SELKER 1990). Therefore, we expect that in many progeny both of the *cya-8*^{RIP} copies are methylated. Spread of methylation from the ectopic *cya-8*^{ecRIP} region into the adjoining promoter region of *eas* (short arrow) was detected by Southern analysis (see Figure 2), strongly suggesting that RIP is responsible for the recurrent mutations at *cya-8*.

following transformation. Epigenetic alterations due to quelling are unstable, in contrast to the stability of *eas*^{UCLA}-induced *cya-8* mutations. We failed to find mutations to *cya-8* in vegetative cultures from *eas*^{UCLA} conidia. To be responsible for the unstable transparent germinants produced by *eas*^{UCLA}, quelling would need to occur preferentially at the time of ascospore germination, which seems highly unlikely.

2. MSUD is transitory and is not known to produce stable mutations (SHIU *et al.* 2001). Our experiments with the *Sad-2* suppressor revealed that MSUD is not involved in the production of stable transparent progeny (Table 6).
3. Loss of mitochondrial function is not a tenable explanation. Although the phenotype of the transparent strains resembles that of mitochondrial respiratory mutants produced by a mutator described in Saccharomyces by EVANS and WILKIE (1978), the *eas*^{UCLA}-induced transparent progeny in Neurospora are due to the single Mendelian mutation *cya-8*, which is transmitted to progeny regardless of whether the *eas*^{UCLA} parent is male or female. Mitochondrial inheritance is strictly maternal in Neurospora, through the protoperithecial parent. The mitochondrial genome of the fertilizing parent is not transmitted through crosses (MITCHELL *et al.* 1953; MANNELLA *et al.* 1979).
4. A mobile element is probably not involved. Only one active transposable element is known in any strain of Neurospora, the retrotransposon *Tad* (KINSEY and HELBER 1989). *Tad* proliferates in vegetative cultures, but when strains are crossed, the copies are inactivated by RIP (KINSEY *et al.* 1994). Active *Tad* elements are absent in the wild-type strains used in this study.

The recurrent appearance of *cya-8* mutations in crosses with *eas*^{UCLA} is superficially similar to what is seen with the *Dotted* mutator in maize, which produces red dots on an unpigmented background in the kernel

(Rhoades 1948). *Dotted* was shown by McClintock (1950) to be identical to the controlling element *Activator* (*Ac*), and the induced gain of anthocyanin pigmentation in the mutant areas is due to relief of inhibition by excision of the nonautonomous transposable element *Dissociator* (*Ds*) from the a_1 locus. *Ds* moves only when transposase is provided by *Ac*, and this is precisely timed during development. Like eas^{UCLA} and *cya-8*, *Ds* and *Ac* show locus specificity for the target gene. Suggestive examples of transposon-induced recurrent mutation have been found in the Discomycete *Ascobolus immersus*, with instability of ascospore color attributed to a mobile element that is excised premeiotically (Decaris *et al.* 1981; Nicolas *et al.* 1987). No active two-element DNA transposable element system has been identified in Neurospora, however.

5. Deletion resulting from intrachromosomal recombination cannot be responsible for the *cya-8* mutations. Behavior of eas^{UCLA} differs in several respects from that of *buf1*, a mutable gene in another Pyrenomycete, *Magnaporthe grisea*, with which Chumley and Valent (1990) and Farman (2002) have described a process called meiosis-associated deletion in heteroallelic repeats (MDHR). In certain crosses, 5–25% of progeny have acquired new *buf1* mutations. These are all deletions that result from intrachromosomal recombination. Ascus analysis shows that, when deletion occurs, both chromatids of a chromosome are usually affected, indicating that the mutations occur prior to premeiotic DNA replication. Unlike other phenotypically normal *buf1* alleles that are stable, the unstable *buf1* allele contains numerous repetitive elements. The MDHR mutations occur predominantly in heteroallelic crosses, suggesting that failure of pairing may promote the recombination event that leads to deletion.

The detection of inversions: The eas^{UCLA} strain analyzed here provides the first well-studied example of a paracentric inversion in Neurospora. The only other documented case is an ~20-kb inversion detected by DNA sequencing (Micali *et al.* 2001; Micali and Smith 2006).

Most of the known chromosome rearrangements in Neurospora have been detected because inviable, unpigmented ascospores are produced when the rearrangement is heterozygous. The defective ascospores result from deficiencies that are generated by meiotic assortment and recombination. Unpigmented, aborted ascospores are produced abundantly by heterozygous reciprocal and insertional translocations. Long pericentric inversions also generate enough deficiency ascospores to be recognized in this way (see, for example, Newmeyer and Taylor 1967; Turner *et al.* 1969; Barry and Leslie 1982; Turner and Perkins 1982). The method fails to detect paracentric inversions, however. Thus, the eas^{UCLA} inversion, which is unrecognizable by the criterion of aborted-ascospore production, was discovered only because progeny that have undergone crossing over in a segment adjoining the *eas* locus are absent when eas^{UCLA} is heterozygous. The inversion was confirmed by showing that gene order is reversed and crossing over is no longer blocked when the mutant is homozygous (Table 8).

Synaptonemal-complex reconstructions (Bojko 1990) and orcein-stained squashes (Barry and Leslie 1982) have been used to demonstrate that heterozygous long pericentric inversions pair homologously, forming loops at pachytene. Synaptic adjustment follows, leading to disappearance of the loop (Bojko 1990). When the inverted segment is short, homologous pairing may not occur or may not be cytologically visible. Even if pairing occurs, crossing over may be too infrequent to produce enough defective ascospores to distinguished from the 5 or 10% noise level that is characteristic of crosses between highly inbred, presumably isosequential strains. Perkins and Barry (1977) have speculated that, even if pairing were effective and single crossovers occurred with an appreciable frequency in a heterozygous paracentric inversion, the dicentric bridges that were produced might result in the death and resorption of all asci. Their loss would preclude the production of enough unpigmented ascospores to provide a signal for detection.

Short inversions and other small rearrangements were shown to be at least as common as gross rearrangements in differentiating the genome of *Saccharomyces cerevisiae* from that of *Candida albicans* (Seoighe *et al.* 2000). It is reasonable to suggest that paracentric inversions may be present as undetected genetic polymorphisms in laboratory stocks and wild populations of Neurospora.

Complex rearrangements: If our diagnosis is correct, eas^{UCLA} originated as a combination paracentric inversion and insertional translocation (Figure 3). The rearrangement had four breakpoints, one of which is likely shared between the inversion and the translocation.

Rearrangements with four or more breakpoints are not uncommon in Neurospora. Their presence is readily recognized when multiple linkage groups are involved, because <50% of ascospores are viable in crosses where a multibreak complex rearrangement is heterozygous. Genetic analysis of multibreak rearrangements is laborious. For this reason, most putative complex rearrangements have been set aside without being investigated further. A few illustrative examples have been thoroughly analyzed, however. Among these are *In(IL;IR) T(IL;IIIR)SLm-1*, in which an inversion and a reciprocal translocation have one breakpoint in common (Barry 1992); *T(IVR → VIIL;IL;IIR;IVR)S1229*, in which insertional and reciprocal translocations share a common breakpoint (Barry 1960); and *Tp(IR→IL)T54M94*, an inverted insertion having multiple breaks in the same chromosome (Perkins *et al.* 1995). Other complex Neurospora rearrangements are described by Perkins (1997).

A four-break rearrangement in the laboratory mouse resembles what we infer to have occurred with *eas*^{UCLA}: Rearrangement *Is(17;In2)1Gso* has a segment of mouse chromosome 17 inserted at one of the breakpoints of a chromosome 2 inversion (Beechey and Evans 1996; Lyon *et al.* 1996). If Neurospora conventions were used, this mouse rearrangement would be symbolized as *In(2)T(17→2)Gso.* Complex rearrangements in other eukaryotes may be far more common than has been revealed by classical cytogenetic methods (Savage 2002).

Nomenclature: By convention, the hypothesized original *eas* rearrangement would be symbolized as *In(IIR) T(VIIL → IIR)UCLA191 eas.* In the derived strains that were retained and that were used here, the original linkage group VII donor with its deficiency has been replaced by a normal-sequence chromosome. Their genotype would be symbolized *In(IIR)Dp(VIIL → IIR) UCLA191 eas.* Extent of the inserted segment is unknown and could be as short as a single gene. In practice, the insertion is cryptic and can usually be ignored. In contrast, effects of the inversion on recombination in LG IIR are clearly manifested, especially when it is heterozygous.. Existing strains can be used as though the *eas*^{UCLA191} mutation were a simple paracentric inversion, and this may be useful not only for investigating the behavior of paracentric inversions but also as a balancer or crossover suppressor. We propose to adopt the shortened symbol *In(IIR)UCLA191 eas* for the rearrangement called *eas*^{UCLA} in this article.

The *eas*^{UCLA}*-cya-8* system as a model: The experiments reported here support the following hypothesis: When a gene (the "donor") is inserted ectopically at the locus of another gene (the "recipient") and the recipient gene is simultaneously mutated, the mutant complex then has the potentiality of acting as a locus-specific mutator of a wild-type donor gene. Mutation results from RIP and it will occur in crosses where a second copy of the donor gene is present in its normal position in the same nucleus with the ectopic mutator complex. The mutator appears to target the normal allele of the donor gene. The *eas*^{UCLA} strain has provided the first example of this behavior, with a copy of the *cya-8*$^{+}$ gene from linkage group VII inserted at the *eas* locus in linkage group II and with *cya-8*$^{+}$ present at the original locus in linkage group VII (Figure 3). Allele *eas*^{UCLA}, which in reality is a fused complex, *eas cya-8*$^{(EC)}$, thus appears to act as a mutator that is specific for *cya-8*$^{+}$.

Mediation of locus-specific mutation in this way is probably not limited to *eas*^{UCLA}, where it happened to be discovered. When one parent in a cross has the "target" gene transposed and inserted at the locus of the putative "mutator," genes other than *eas* and *cya-8* should be capable of behaving as mutator/target pairs in Neurospora. Transposition of the target sequence may be induced or may occur spontaneously. Segmental rearrangements that involve transposition and ectopic insertion of essential genes are not uncommon in Neurospora, where they are readily recognized (Perkins 1997). Ectopic insertion of transforming DNA sequences is typical in Neurospora. Now that DNA sequences and the necessary molecular-genetic tools are available, it should be possible, with *eas*^{UCLA}*-cya-8* as an example, to construct other mutator systems using any two well-characterized loci. A locus-specific mutator system of this type might well involve only a simple single-gene insertion without any additional complexity such as the IIR inversion in *eas*^{UCLA}, which can be considered a fortuitous distraction.

Genetic analysis was made by David Perkins with the assistance of Virginia Pollard. Molecular analyses were carried out by Michael Freitag and Eric Selker. The mutant *fluffy* allele was inserted in *eas*^{UCLA} by Lori Bailey-Schrode and Dan Ebbole. *rid; eas*^{UCLA} strains were constructed by Michael Freitag. We are grateful to Helmut Bertrand for his characterization of *cya-8* and to Edward Barry and N. B. Raju for microscopic observations of meiosis. Kohji Hasanuma provided *eas* allele KH5-9, and Jay Dunlap provided allele JD105. Patrick Shiu and Robert Metzenberg provided *Sad-2* strains. Deborah Bell-Pedersen provided pS7C1. Frank Lauter contributed helpful information prior to publication. The work was supported by research grants from the National Science Foundation (MCB-0417282 to D.D.P., MCB-0131383 to E.U.S., and MCB-9974608 to D.J.E.) and the National Institutes of Health (AI01462 to D.D.P. and GM35690 to E.U.S.).

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