REFERENCES AND NOTES

1. R. E. Kendrick and G. H. M. Kronenberg, Eds., *Photomorphogenesis in Plants* (Kluwer Academic, Dordrecht, Netherlands, 1994).
2. B. M. Sweeney, *Rhythmic Phenomena in Plants* (Academic Press, San Diego, CA, 1987); L. N. Edmunds, *Cellular and Molecular Bases of Biological Clocks* (Springer-Verlag, New York, 1988).
3. B. A. Horwitz and B. L. Epel, *Plant Sci. Lett.* **13**, 9 (1978).
4. E. Simon, R. L. Satter, A. W. Galston, *Plant Physiol.* **58**, 421 (1976).
5. T. Roenneberg and J. W. Hastings, *Naturwissenschaften* **75**, 206 (1988); J. C. Harris and M. B. Wilkins, *Planta* **129**, 253 (1976).
6. G. Bernier, A. Havelange, C. Houssa, A. Petitjean, P. Lejeune, *Plant Cell* **5**, 1147 (1993).
7. C. R. McClung, S. A. Kay, in Arabidopsis thaliana, C. S. Somerville and E. Meyerowitz, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1994).
8. A. J. Millar, S. R. Short, N.-H. Chua, S. A. Kay, *Plant Cell* **4**, 1075 (1992); A. J. Millar, Ph.D. thesis, Rockefeller University (1994).
9. Imaging conditions and *Arabidopsis* lines carrying *cab2*::Ω::*Luc* have been described [A. J. Millar, S. R. Short, K. Hiratsuka, N.-H. Chua, S. A. Kay, *Plant Mol. Biol. Rep.* **10**, 324 (1992)].
10. Seeds for in vivo imaging were sown in a grid pattern and stored for 4 to 7 days in dim light at 4°C. Seedlings were grown for 5 days in a controlled environment chamber at 22°C, under 50 to 60 μmol m^{-2} s^{-1} fluorescent light in LD. At dawn on the sixth day of growth, the seedlings were transferred to constant conditions and imaged as described (*9*).
11. For estimating the period, we used a modified sine wave function of the form

$$L(t) = (c_0 + c_1 t) + (amp_0 + amp_1 t)\sin(2\pi t/T - \phi)$$

where L is luminescence, t is time, T is the period estimate, and ϕ is the estimate of the phase at $t = 0$. The variable c_0 is the estimated value of the luminescence level at $t = 0$, and c_1 is an estimate of the linear rate of change in luminescence level; amp_0 is the estimated value of the cycling amplitude at $t = 0$, and amp_1 is an estimate of the linear rate of change in cycling amplitude. The fitting is based on nonlinear least squares minimization of the variance of fit. Variance-weighted means of period estimates ($\langle T \rangle$, or <per> in Tables 1 and 2) within a group of seedlings were calculated according to Bevington [P. R. Bevington, *Data Reduction and Error Analysis for the Physical Sciences* (McGraw-Hill, New York, 1969), p. 73] and variance-weighted standard deviations by a similar method. Full details of these programs are available from the authors. Significance levels were taken as 5%.
12. Red light had a λ_{max} of 660 nm and a half-bandwidth of 15 nm, and blue light had a λ_{max} of 460 nm and a half-bandwidth of 60 nm, at 23 to 25 μmol m^{-2} s^{-1}.
13. A. J. Millar and S. A. Kay, unpublished results.
14. J. Chory *et al.*, *Plant Cell* **1**, 867 (1989).
15. A. Pepper, T. Delaney, T. Washburn, D. Poole, J. Chory, *Cell* **78**, 109 (1994).
16. J. Chory, P. Nagpal, C. Peto, *Plant Cell* **3**, 445 (1991).
17. X.-W. Deng, T. Caspar, P. H. Quail, *Genes Dev.* **5**, 1172 (1991); T. W. McNellis *et al.*, *Plant Cell* **6**, 487 (1994).
18. Wild-type Columbia-1 and *cop1-6* were provided by G. P. Rédei (University of Missouri–Columbia).
19. J. Chory, *Trends Genet.* **9**, 167 (1993); X.-W. Deng, *Cell* **76**, 423 (1994).

20. We thank K. W. Smith for assistance, D. Gerber for imaging, D. Somers for critically reading the manuscript, and T. M. Breeden for programming. Supported by grants from the NSF Center for Biological Timing to S.A.K. and NIH grant GM44640 to N.-H.C. A.J.M. was supported by a William O. Baker Fellowship, through a grant from the Mellon Foundation. S.A.K. is supported by an award from the W. M. Keck Foundation.

8 September 1994; accepted 6 December 1994

A Role for Exonuclease I from *S. pombe* in Mutation Avoidance and Mismatch Correction

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Exonuclease I (Exo I) from *Schizosaccharomyces pombe*, a 5′ → 3′ double-stranded DNA exonuclease, is induced during meiotic prophase I. The *exo1* gene is a member of a family of related DNA repair genes, including *RAD2/rad13/xpgc* and *YKL510/rad2*, conserved from yeast to humans. An *exo1* mutant displays a mutator phenotype and alters activity of the *ade6-M387* marker effect. These results suggest that Exo I acts in a pathway that corrects mismatched base pairs.

Exonucleases act in multiple pathways of DNA repair and recombination. Enzymes removing only one strand of duplex DNA are postulated to create single-stranded DNA (ssDNA) that, in turn, can act as a substrate for homologous pairing and strand-exchange activities during recombination and recombinational repair of broken chromosomes (*1*, *2*). *Escherichia coli* and its phages encode such strand-specific exonucleases; strains lacking these enzymes are deficient in various aspects of recombination and repair (*1*). In eukaryotes, identified intermediates in double-strand break repair are consistent with the action of 5′ → 3′ double-stranded DNA (dsDNA) exonucleases (*3*).

Exonucleases are also implicated in the late steps of homologous recombination, namely the correction of mismatched base pairs resulting from hybrid DNA formation between single stands of chromosomes carrying different point mutations. Mismatch correction influences the outcome of recombination in two-factor intragenic crosses. Close genetic markers are often covered by a single tract of heteroduplex DNA and can form recombinants only if the two resulting mismatches are corrected independently. In addition, gradients of gene conversion frequencies across the *ARG4* and *HIS4* genes of *Saccharomyces cerevisiae* are at least in part a result of mismatch correction (*4*, *5*). In *E. coli*, the correction of mismatches caused by replication errors involves the combined action of helicases and ssDNA exonucleases (*6*), but no eukaryotic exonucleases acting in these processes have been identified yet.

We have previously described the activity of a meiotically induced 5′ → 3′ dsDNA exonuclease, Exo I, from *S. pombe* (*7*). Exo I acts on both double-strand ends and nicks and is a good candidate for an activity required in the processes discussed above. Here, we present evidence that Exo I contributes to mismatch correction.

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The *exo1* gene was cloned by polymerase chain reaction with degenerate primers that were derived from a peptide sequence of the Exo I protein (*8*). From the 2-kb insert of one of the *exo1* clones, pXON4, the DNA sequence of both strands was determined (GenBank accession number L35174). A single, uninterrupted open reading frame (ORF) encoding a putative polypeptide of 63,866 daltons was identified (*9*). The size of purified Exo I was estimated by SDS–polyacrylamide gel electrophoresis to be approximately 36,000 daltons (*7*). We presume that previously purified Exo I is a proteolytic product.

By comparison of the predicted Exo I amino acid sequence to those in databases, we found it was homologous to that of previously reported proteins (Fig. 1). The *S. cerevisiae RAD2* gene, which encodes a ssDNA endonuclease, and its *S. pombe* and human homologs *rad13* and *xpgc*, respectively, all function in ultraviolet (UV) excision repair (*10–12*). Although these genes are assumed to encode functional homologs (*13*), the strong sequence homologies are limited to discrete blocks interspersed with regions of poor conservation (*14*). The *exo1* gene is a composite of some of these blocks but with less sequence conservation than between *RAD2*, *rad13*, and *xpgc*. Another *S. pombe* gene involved in UV excision repair, *S.p. rad2* (*15*, *16*), and its presumed homologs from *S. cerevisiae*, *YKL510* (*17*), and from humans, *H.s. rad2* (*16*), have the same composite structure as *exo1* but are much more closely related to each other than to *exo1* (Fig. 1). The *exo1* gene appears to represent a new, third class in this family of related genes.

To explore the basis of the rapid rise and decrease of enzyme activity during meiosis (*7*), we carried out a Northern (RNA) analysis of RNA isolated from cells at different points during meiosis (Fig. 2A). At 4, 5, and 6 hours after induction of meiosis, the amount of *exo1* transcript increased approximately 10-fold, peaking at 5 hours. This increase roughly parallels the changes detected in dsDNA exonuclease activity detected in cell extracts (*7*) and suggests con-

trol of Exo I activity at the RNA level.

We constructed an *exo1* mutant by replacing a Sty I to Bgl I fragment (containing 48 base pairs upstream of the ORF and 326 codons out of 571) of *exo1* with the *ura4* gene (9). Strains carrying the resulting *exo1-1::ura4* mutation were not detectably affected in conjugation, sporulation, or spore germination. We confirmed disruption of the *exo1* gene by measuring dsDNA exonuclease activity during a meiotic time course of a strain carrying the *exo1-1::ura4* replacement (Fig. 2B). The enzyme activity during both mitosis and meiosis was significantly reduced, demonstrating that the mitotic and meiotic activities measured in the wild-type strain are indeed a result of the action of the same enzyme.

We noticed that the *exo1-1::ura4* mutation caused the frequent appearance of white colonies in *ade6*$^-$ strains that normally form red colonies on plates containing limiting amounts of adenine. These white segregants were still Ade$^-$ and were probably the result of mutation of "early" *ade* genes whose products act before *ade6*. To quantitate this mutator phenotype, we measured the reversion of two point mutations (Table 1). The *exo1-1::ura4* mutation caused a 17- to 26-fold increase in the reversion rate of *ade7-C8* (an ICR-170–induced mutation of unknown type) and a 4- to 13-fold increase in the reversion rate of *ade6-M26* (a G→T transversion). The *exo1* gene could be allelic with the previously characterized *mut2* gene, because both genes map to the same Not I fragment and cause a comparable mutator effect when mutated (9, *18*).

Spontaneous mutations may occur when replication errors form mismatched base pairs that escape correction before the next S phase. We analyzed meiotic recombination in short intragenic intervals to determine whether Exo I also plays a role in the correction of mismatched base pairs in hybrid DNA produced during meiotic recombination. In intragenic crosses, close markers yield very low recombinant frequencies because the average lengths of hybrid DNA and mismatch correction tracts exceed the distance between the two markers and produce predominantly co-conversions. But if the alleles involved can form C:C mismatches, which are poorly repaired (*19*), recombinant frequencies are strongly elevated (*20*, *21*). This is probably a result of an increase in single-site correction caused by a second, competing short-patch correction pathway that recognizes C:C mismatches. In accordance with genetic evidence, *S. pombe* has two distinct mismatch binding activities (*22*), one binding to every mismatch except C:C, the other binding to all mismatches that contain C, including C:C.

We conducted crosses between closely spaced alleles of the *ade6* gene (Table 2). In *exo1*$^+$ strains, *ade6-M387* (which can form C:C mismatches) yielded 10- to 20-fold greater recombinant frequencies than the nearby allele *ade6-51* when it was crossed to *ade6-485* or to *ade6-442* (Table 2). The *ade6-485* allele also creates C:C mismatches

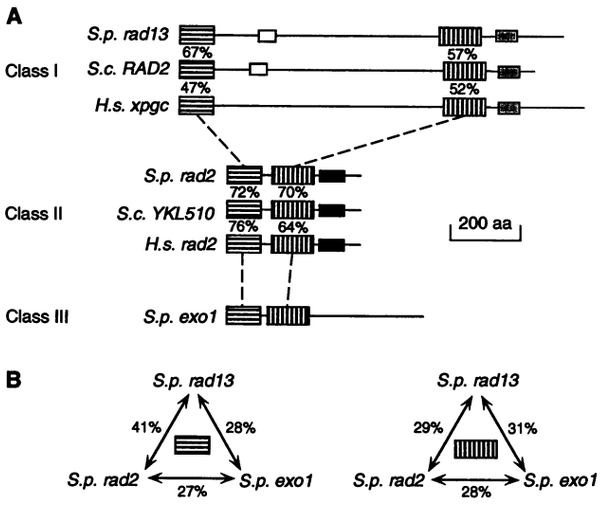

Fig. 1. Genes related to *exo1*. Homologies to Exo I were detected in a FASTA search (*31*) of databases at the National Center for Biotechnology Information and further characterized with the use of BESTFIT (*31*). (**A**) The domains of strongest homology among all proteins are diagrammed as large, striped boxes. Additional regions highly conserved among a subset of proteins are diagrammed as small boxes. Numbers between the sequences within classes I and II represent the percentage of identical amino acids within the respective domains. *H.s., Homo sapiens*; *S.c., Saccharomyces cerevisiae*; *S.p., Schizosaccharomyces pombe*; aa, amino acids. (**B**) Numbers indicate the percentage of identity among the three *S. pombe* proteins for the two conserved domains.

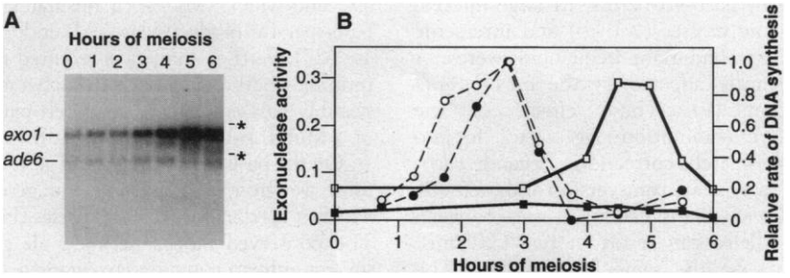

Fig. 2. Regulation of *exo1* during meiosis. Synchronous meiosis was induced in strains harboring the conditional *pat1-114* allele as described (*7*). (**A**) Total RNA was prepared from cells of strain GP972 (*h*$^+$ *pat1-114 end1-458 ura4-D18 leu1-32 ade6-M26*) harvested at the times indicated. From each time point, 5 μg of RNA was used for Northern analysis with the insert of pXON4 and a 2-kb Xho I fragment of *ade6* (*32*) as hybridization probes. Positions of the ribosomal RNAs are indicated by asterisks. (**B**) In strains GP1083 (*h*$^-$ *exo1-1::ura4 pat1-114 end1-458 ura4-D18 leu1-32*) and GP535 (*h*$^-$ *pat1-114 end1-458 ade6-M26*), exonuclease activity and premeiotic DNA synthesis were measured as described (*7*). Exonuclease activity is given as enzyme units per milligram of protein in crude extracts (squares and thick lines) (*7*). Premeiotic DNA synthesis is given as relative incorporation of ^{3}H from [^{3}H]uracil into DNA per time interval (circles and dashed lines) (*7*). Filled symbols, GP1083 (*exo1-1::ura4*); open symbols, GP535 (*exo1*$^+$). Data for strain GP535 are taken from (*7*).

Table 1. Increased reversion rates in an *exo1* mutant. For each strain, five cultures [in yeast extract liquid (YEL) plus adenine] were each inoculated with an independent colony. From each culture, approximately 10^8 cells were plated on a total of 10 yeast extract agar (YEA) plates containing guanine (200 μg/ml) for selection of adenine prototrophs (*33*). Reversion rates were calculated by the method of the median (*34*). Complete genotypes and genealogies are available on request.

Strain	Relevant genotype	Reversion rate (per 10^8 cell divisions)	Fold increase
GP1383	*ade7-C8*	1.2 ± 0.6	1
GP1381	*ade7-C8 exo1-1::ura4*	32 ± 7.1	27
GP1382	*ade7-C8 exo1-1::ura4*	21 ± 4.9	18
GP1143	*ade6-M26*	2.7 ± 1.1	1
GP1144	*ade6-M26*	2.1 ± 0.9	1
GP1153	*ade6-M26 exo1-1::ura4*	11 ± 3.3	4
GP1154	*ade6-M26 exo1-1::ura4*	27 ± 6.7	13

Table 2. Alteration of the *ade6-M387* marker effect in an *exo1* mutant. Strains were crossed and spores were plated on YEA and YEA plus guanine (200 μg/ml) for total and adenine prototrophic spores, respectively, as described (*33*). Crosses homozygous for each *ade6* allele showed that reversion rates did not influence the apparent recombinant frequencies (*9*). Complete genotypes and genealogies are available on request. Point mutations and numbering have been described (*20, 32*). Average recombinant frequencies were derived from the independent measurements from separate crosses that are shown in parentheses.

Genotype	Meiotic recombinant frequency (Ade$^+$ recombinants/10^6 spores) when crossed with	
	ade6-485 (C2119→G)	*ade6-442* (ΔT2097)
	exo1$^+$/*exo1*$^+$	
ade6-51 (C2141→T)	16* (8, 13, 27)	1.3 (1, 1, 2)
ade6-M387 (G2145→C)	194 (140, 200, 242)	29 (27, 29, 30)
	exo1-1::ura4/exo1-1::ura4	
ade6-51	42 (33, 42, 52)	8 (6, 10)
ade6-M387	32 (24, 34, 39)	56 (51, 52, 64)
	exo1-1::ura4/exo1$^+$	
ade6-51	5 (4, 5, 6)	2.7 (2, 2, 4)
ade6-M387	109 (73, 126, 127)	31 (26, 27, 41)

and, as expected, yielded higher recombinant frequencies with *ade6-51* than did *ade6-442* (16 versus 1.3 Ade$^+$ recombinants per 10^6 meiotic spores). In the *exo1-1::ura4* background, recombinant frequencies increased two- to sixfold in three sets of crosses and decreased by six times in the *ade6-M387* by *ade6-485* cross. In large-interval intragenic crosses (>1 kb) and intergenic crosses, recombinant frequencies were not significantly affected by the *exo1-1::ura4* mutation (*9*). These effects of the *exo1-1::ura4* mutation suggest a role for Exo I in mismatch correction. Because *ade6-M387* is a G→C transversion and *ade6-485* is a C→G transversion, crosses between these alleles can result in two C:C mismatches on the same heteroduplex. The high recombinant frequencies in crosses between these two alleles may result from mismatch correction by a short patch pathway (*20*). One possible explanation for this result is that the *exo1-1::ura4* mutation abolishes this short patch pathway and thereby decreases this recombinant frequency. On the other hand, an increase in small-interval intragenic recombinant frequencies (observed for markers involving one or no C:C mismatch) could be caused by the absence of a long patch mismatch correction pathway in the *exo1-1::ura4* mutant (*23*).

Because Exo I can degrade one strand in the 5′ → 3′ direction starting at a nick (*7*), we propose that it acts after the introduction of a nick 5′ to a mismatched base pair. This mechanism contrasts with that of *dam* methylation-directed mismatch correction in *E. coli*, in which one strand is degraded by the combined action of a helicase and ssDNA exonucleases (*6*). Components of the bacterial *dam* methylation-directed mismatch correction pathway, namely MutL and MutS, are conserved in yeast and mammals (*5, 24, 25*), but the mode of nucleotide excision in eukaryotic mismatch correction is unknown. In *E. coli*, two systems for correction of mismatches by short patch repair that are independent of *dam* have been characterized (*26*). They involve Vsr, a sequence- and strand-specific endonuclease, and MutY (MicA), a mismatch- and base-specific N-glycosylase–AP-endonuclease (*27*). Further analysis is required to determine whether Exo I acts in such a minor, possibly base-specific, short patch pathway or a MutHLS-like long patch pathway.

On the basis of homology and gene structure, we grouped *exo1* and the genes to which it is related into three classes (Fig. 1). The conserved blocks between all classes suggest either a common enzymatic activity, such as an exonuclease and endonuclease, or a common binding partner, such as a class of proteins that bind to sites of DNA damage. Despite the relation of *exo1* to UV excision repair genes, there was no significant effect of the *exo1-1::ura4* mutation on UV or gamma ray sensitivity (*9*). In humans the lack of homologs of MutL and MutS is associated with hereditary nonpolyposis colon cancer and instability of oligonucleotide repeats (*25, 28*). A homolog of Exo I in multicellular eukaryotes may also play a pivotal role in maintaining genome stability.

REFERENCES AND NOTES

1. G. R. Smith, *Microbiol. Rev.* **52**, 1 (1988); S. C. Kowalczykowski, D. A. Dixon, A. K. Eggleston, S. D. Lauder, W. M. Rehrauer, *ibid.* **58**, 401 (1994).
2. T. D. Petes, R. E. Malone, L. S. Symington, in *The Molecular and Cellular Biology of the Yeast* Saccharomyces, J. R. Broach, J. R. Pringle, E. W. Jones, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1991), vol. 1, pp. 407–521.
3. E. Maryon and D. Carroll, *Mol. Cell. Biol.* **9**, 4862 (1989); L. Cao, E. Alani, N. Kleckner, *Cell* **61**, 1089 (1990); C. I. White and J. E. Haber, *EMBO J.* **9**, 663 (1990); H. Sun, D. Treco, J. W. Szostak, *Cell* **64**, 1155 (1991).
4. P. Detloff, M. A. White, T. D. Petes, *Genetics* **132**, 113 (1992).
5. R. A. Reenan and R. D. Kolodner, *ibid.*, p. 963.
6. R. S. Lahue, K. G. Au, P. Modrich, *Science* **245**, 160 (1989).
7. P. Szankasi and G. R. Smith, *J. Biol. Chem.* **267**, 3014 (1992).
8. Exo I protein [approximately 200 pmol of fraction V (*7*)] was transferred to a nitrocellulose membrane, stained, and excised according to recommendations by W. S. Lane (Harvard MicroChem, Harvard University), who carried out the subsequent internal amino acid sequence analysis. From the 22–amino acid sequence obtained (EHGIESIVAPYEADAQLVYLEK; abbreviations for the amino acid residues are: A, Ala; D, Asp; E, Glu; G, Gly; H, His; I, Ile; K, Lys; L, Leu; P, Pro; Q, Gln; S, Ser; V, Val; and Y, Tyr), two degenerate primers (IA: 5′-GAA/GCAC/TGGNATA/C/TGA-3′; and IB: 3′-ATA/GCTC/TCGNCTA/GCGNGTC/T-5′) corresponding to opposite strands were derived. With one or the other primer labeled with ^{32}P at the 5′ end, a low-stringency polymerase chain reaction (*29*) was carried out on genomic DNA of *S. pombe*. After elution from a urea-polyacrylamide gel, the product was sequenced according to the chemical degradation method (*30*). From this sequence, a 38-nucleotide primer (IC: 5′-GGGATTGAATCAATCGTTGC-TCCGTACGAAGCCGACGC-3′) was obtained and used to screen a genomic library of *S. pombe* by colony hybridization.
9. P. Szankasi and G. R. Smith, unpublished results.
10. R. J. Reynolds and E. C. Friedberg, *J. Bacteriol.* **146**, 692 (1981); D. R. Wilcox and L. Prakash, *ibid.* **148**, 618 (1981).
11. K. Madura and S. Prakash, *ibid.* **166**, 914 (1986); D. Scherly *et al.*, *Nature* **363**, 182 (1993).
12. Y. Habraken, P. Sung, L. Prakash, S. Prakash, *Nature* **366**, 365 (1993).
13. S. J. McGready, H. Burkill, S. Evans, B. S. Cox, *Curr. Genet.* **15**, 27 (1989).
14. A. M. Carr *et al.*, *Nucleic Acids Res.* **21**, 1345 (1993).
15. J. Phipps, A. Nasim, D. R. Miller, *Adv. Genet.* **23**, 1 (1985).
16. J. M. Murray *et al.*, *Mol. Cell. Biol.* **14**, 4878 (1994).
17. A. Jacquier, P. Legrain, B. Dujon, *Yeast* **8**, 121 (1992).
18. P. Munz, *Mutation Res.* **29**, 155 (1975).
19. J.-P. Claverys, V. Méjean, A.-M. Gasc, A. M. Sicard, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5956 (1983); J. H. White, K. Lusnak, S. Fogel, *Nature* **315**, 350 (1985); P. Schär, P. Munz, J. Kohli, *Genetics* **133**, 815 (1993).
20. P. Schär and J. Kohli, *Genetics* **133**, 825 (1993).
21. C. W. Moore, D. M. Hampsey, J. F. Ernst, F. Sherman, *ibid.* **119**, 21 (1988).
22. O. Fleck, P. Schär, J. Kohli, *Nucleic Acids Res.*, in press.
23. M. S. Williamson, J. C. Game, S. Fogel, *Genetics* **110**, 609 (1985).
24. W. Kramer, B. Kramer, M. S. Williamson, S. J. Fogel, *J. Bacteriol.* **171**, 5339 (1989); T. A. Prolla, D. M. Christie, R. M. Liskay, *Mol. Cell. Biol.* **14**, 407 (1994).
25. R. Fishel *et al.*, *Cell* **75**, 1027 (1993); F. S. Leach *et al.*, *ibid.*, p. 1215; C. E. Bronner *et al.*, *Nature* **368**, 258 (1994); N. Papadopoulos *et al.*, *Science* **263**, 1625 (1994).
26. M. Lieb, E. Allen, D. Read, *Genetics* **114**, 1041 (1986); K. G. Au, M. Cabrera, J. H. Miller, P. Modrich, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9163 (1988).
27. F. Hennecke, H. Kolmar, K. Bründl, H.-J. Fritz, *Nature* **353**, 776 (1991); J. J. Tsai-Wu, H. F. Liu, A. L. Lu, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8779 (1992).
28. S. N. Thibodeau, G. Bren, D. Schaid, *Science* **260**, 816 (1993); Y. Ionov, M. A. Peinado, S. Malkhosyan, D. Shibata, M. Perucho, *Nature* **363**, 558 (1993).
29. T. Compton, in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, San Diego, 1990), pp. 39–45.
30. J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989).
31. J. Devereux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984).
32. P. Szankasi, W.-D. Heyer, P. Schuchert, J. Kohli, *J. Mol. Biol.* **204**, 917 (1988).

33. Standard genetic techniques and media were as described or referenced [L. C. DeVeaux, N. A. Hoagland, G. R. Smith, *Genetics* **130**, 251 (1992); C. Grimm, J. Bähler, J. Kohli, *ibid.* **136**, 41 (1994)].
34. D. E. Lea and C. A. Coulson, *J. Genet.* **49**, 264 (1949).
35. We thank M. Roth, J. Ranish, and W. Wahls for advice in cloning; L. Clarke at the University of California, Santa Barbara, for the *S. pombe* genomic library; O. Fleck, P. Schär, and J. Kohli for making their data available before publication; P. Munz and J. Kohli for yeast strains; J. Kohli for discussion on mismatch correction; and S. Amundsen, P. Dabert, Y. F. Li, A. Taylor, J. Virgin, and W. Wahls for comments on the manuscript. Supported by NIH grant GM32194 (G.R.S.). The Biocomputing Resource Center at the Fred Hutchinson Cancer Research Center is supported by National Cancer Institute award P30 CA15704-20.

15 August 1994; accepted 14 December 1994

PER Protein Interactions and Temperature Compensation of a Circadian Clock in *Drosophila*

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The periods of circadian clocks are relatively temperature-insensitive. Indeed, the per^L mutation in the *Drosophila melanogaster period* gene, a central component of the clock, affects temperature compensation as well as period length. The *per* protein (PER) contains a dimerization domain (PAS) within which the per^L mutation is located. Amino acid substitutions at the per^L position rendered PER dimerization temperature-sensitive. In addition, another region of PER interacted with PAS, and the per^L mutation enhanced this putative intramolecular interaction, which may compete with PAS-PAS intermolecular interactions. Therefore, temperature compensation of circadian period in *Drosophila* may be due in part to temperature-independent PER activity, which is based on competition between inter- and intramolecular interactions with similar temperature coefficients.

Circadian rhythms, nearly ubiquitous in eukaryotes, are self-sustaining, have periods of approximately 24 hours, and can be entrained by environmental signals such as light. Unlike many physiological processes that are dependent on temperature, the period lengths of circadian clocks are nearly constant over a wide range of physiological temperatures (*1*). Such temperature compensation is crucial to the construction of a circadian clock; otherwise, the period length of the clock would fluctuate with ambient temperature and would be unreliable.

Although a number of hypotheses have been proposed (*2*), there is no experimental information on the molecular basis of temperature compensation in any system. However, mutations that compromise temperature compensation exist in the *D. melanogaster period (per)* gene and in the *Neurospora frequency (frq)* gene (*3–5*). For example, the period length of wild-type *Drosophila* varies less than 0.5 hour from 15°C to 30°C, but the periods of per^L flies increase from about 27 hours to 33 hours over this temperature range, a change of more than 20% (*4, 6*). Because both *per* and *frq* genes have been shown to be central components of circadian pacemakers (*7*), temperature compensation is likely to derive directly from intrinsic properties of clock components or their interconnection.

The *per* mRNA and protein levels undergo robust circadian oscillations (*8–10*), and there is a feedback loop in which PER affects the circadian transcription of its own gene (*8, 11*). PER contains a PAS domain, a protein dimerization motif present in several basic helix-loop-helix transcription factors (*12, 13*). The per^L mutation is a valine to aspartic acid missense mutation (*14*) in the PAS domain, which affects PAS-mediated PER-PER homodimerization in vitro (*12*) as well as period length and temperature compensation in vivo (*4, 6*).

To perform a structure-function analysis of the PER PAS domain, we reconstituted PER dimerization in a yeast two-hybrid system (Fig. 1) (*15*). Consistent with our previous in vitro results, the large PAS-containing fragment (PER 233–685) dimerized well in this assay (Fig. 2A). Reporter gene activation was dependent on PER interaction, because PER 233–685/LexA with the prey vector alone did not produce any detectable β-galactosidase. The per^S mutation, a serine to asparagine substitution at position 589 that shortens the circadian period from 24 to 19 hours (*3, 14, 16*), dimerized as well (Fig. 2A). Surprisingly, the per^L mutation also showed dimerization nearly indistinguishable from that of the wild-type (Fig. 2A), in contrast to our previous in vitro results that indicated a significant decrease in dimerization (*12*). Because the per^L mutation not only lengthens circadian period but also compromises temperature compensation (*4, 6*), we repeated the yeast assay at higher temperatures. PER^L dimerization was essentially undetectable at 37°C (Fig. 2A), which indicates that some aspect of the interaction is temperature-sensitive.

To substantiate this correlation between circadian behavior in flies and dimerization in yeast, we made another nonconservative change at amino acid 243 (valine to arginine) (PER^LR) and introduced this mutant *per* gene into flies by P element–mediated transformation (*17*). The two independent homozygote mutant lines showed lengthened and temperature-sensitive circadian periods that were very similar to those of the original per^L mutant (Table 1). We also compared the dimerization phenotype of PER^LR in yeast to that of PER^L and of wild-type PER. By growth on a plate without Leu as well as by β-galactosidase activity measurements in liquid media, both the PER^L 233–685 and PER^LR 233–685 bait led to similar decreases in dimerization that were also sensitive to temperature (Table 2). When the preys also carried the same point mutations, dimerization was further reduced and even more sensitive to temperature.

To determine whether the temperature sensitivity of PER^L dimerization might be peculiar to the yeast system, we also tested PER^L dimerization in an in vitro chemical crosslinking and immunoprecipitation assay. PER 233–685/H [which carries a hem-

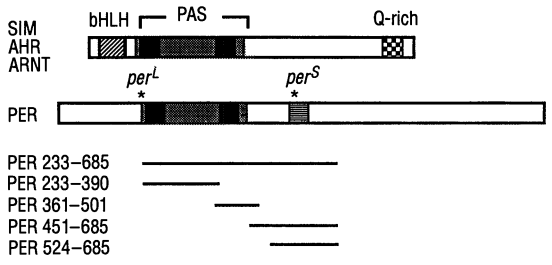

Fig. 1. The basic helix-loop-helix (bHLH) PAS proteins (SIM, AHR, and ARNT) and the full-length PER protein. The bHLH motif, PAS domain, the two 51–amino acid PAS repeats, and the glutamine-rich (Q-rich) region are indicated. The asterisk indicates the per^L and per^S missense mutations. Numbers represent the first and last amino acids of the PER protein fragments. The C-domain is PER 524–685. The shaded box within the C-domain indicates the highly conserved region between fly PER and moth PER (*21*).

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