Acharya, M. R. Botchan, *Science* **270**, 1674 (1995).
9. M. Foss, F. J. McNally, P. Laurenson, J. Rine, *ibid.* **262**, 1838 (1993).
10. S. P. Bell, R. Kobayashi, B. Stillman, *ibid.*, p. 1844.
11. M. Schena, D. Picard, K. R. Yamamoto, *Methods Enzymol.* **194**, 389 (1991).
12. The coding sequence for *DmOrc2* was excised as a 1.9-kb Bsp HI–Sal I fragment from a pNB40-based plasmid [N. H. Brown and F. C. Kafatos, *J. Mol. Biol.* **203**, 425 (1988)] containing the cDNA (*8*), treated with Klenow polymerase and inserted into the Sma I site of pG-3 (*11*) such that the *DmOrc2* transcription start was next to the GPD promoter. This GPD-*DmOrc2*-PGK expression construct was then inserted as a Hind III–Xba I cassette into YIplac128 (*21*) to create pJR1722. The yeast strain JRY4475 (*MATα, HMR-SSΔI, ade2-1, his3-11,15, trp1-1, leu2-3,112, ura3-1, can1-100, orc2-1*) was transformed to leucine prototrophy with Eco RV–linearized pJR1722 [R. J. Klebe, J. V. Harriss, Z. D. Sharp, M. G. Douglas, *Gene* **25**, 333 (1983)].
13. The growth of the *DmOrc2*-expressing *orc2-1* strains JRY5212 and JRY5213 was compared to that of JRY4475 (*orc2-1*) and, as a reference, to the growth of the isogenic *ORC2* strain JRY4473. The strains were streaked on complete medium and incubated at 23°, 26°, 30°, 34°, and 37°C for 3 days, and their growth rates in liquid culture at 23°C were compared.
14. The loss rates (*5*) for plasmids with ARS1 (pJR1469), ARSH4 (pRS316), or the synthetic *HMR*-E silencer (pJR950) as the sole origins of replication were determined in an *ORC2* strain (JRY3009), in an *orc2-1* strain (JRY4475), and in two *DmOrc2*-expressing *orc2-1* strains (JRY5212 and JRY5213) (*5*). The loss rates, expressed as plasmid loss events per cell division, of plasmids replicated by ARS1 or ARSH4 were low (<0.001) in the wild-type strain but were increased in the *orc2-1* strains (0.060 and 0.155 for ARS1 and ARSH4, respectively). The *orc2-1* mutation increased the loss rate for plasmids replicated by the synthetic silencer from 0.024 in the *ORC2* strain to 0.190. The loss rates for *DmOrc2*-expressing *orc2-1* strains were 0.039 ± 0.025, 0.146 ± 0.022, and 0.197 ± 0.069 for ARS1, ARSH4, and the synthetic silencer, respectively.
15. A null allele of *ORC2* was constructed by replacing an internal Msc I fragment of a 5.5-kb Sac I–Sal I *ORC2* fragment by the 1.8-kb Bam HI fragment of *HIS3*. In that *ORC2* is an essential gene, a diploid heterozygous for *orc2-Δ2::HIS3* was constructed by transforming the wild-type diploid JRY4014 to histidine prototrophy with the disruption construct. JRY4014 is a W303-based diploid strain heterozygous for the *HMR-SSΔI* allele. An *ORC2/orc2-Δ2::HIS3* diploid was then transformed with a *URA3*-marked yeast–*E. coli* shuttle vector carrying *ORC2*, pJR1263 (*9*). Tetrads were dissected from the transformed diploid, and a haploid *MATα HMR-SSΔI orc2-Δ2::HIS3* strain containing pJR1263 (JRY5010) was recovered. JRY5010 was transformed to leucine prototrophy with YCplac111 (*21*) containing the GPD-*DmOrc2*-PGK cassette (pJR1720). Several transformants were then tested for their ability to lose the *URA3-ORC2* plasmid by assaying their resistance to 5-fluoroorotic acid (FOA) [J. D. Boeke, F. LaCroute, G. R. Fink, *Mol. Gen. Genet.* **197**, 345 (1984)]. In contrast to JRY5010 cells transformed with a *LEU2-ORC2* plasmid, JRY5010 cells transformed with pJR1720 were unable to grow on FOA, which indicated that they could not lose the *URA3-ORC2* plasmid. Thus, *DmOrc2* expression was unable to rescue the inviability of the *orc2* deletion.
16. K. A. Gavin, M. Hidaka, B. Stillman, *Science* **270**, 1667 (1995).
17. M. Patterson, R. A. Sclafani, W. L. Fangman, J. Rosamond, *Mol. Cell. Biol.* **6**, 1590 (1986).
18. C. Zhou, S. H. Huang, A. Y. Jong, *J. Biol. Chem.* **264**, 9022 (1989).
19. K. S. Weiler and B. T. Wakimoto, *Annu. Rev. Genet.* **29**, 577 (1995).
20. L. Pillus and J. Rine, *Cell* **59**, 637 (1989).
21. R. D. Gietz and A. Sugino, *Gene* **74**, 527 (1988).
22. R. Y. Hampton and J. Rine, *J. Cell Biol.* **125**, 299 (1994).
23. B. J. Brewer and W. L. Fangman, *Cell* **51**, 463 (1987).
24. We thank C. Zucker for providing a clone containing partial *DmOrc2* and alerting us to its existence; C. A. Fox, C. Trueblood, R. Kamakaka, A. Dillin, S. Okamura, and the other members of our laboratories for materials, advice, and discussions; and C. A. Fox, R. Kamakaka, and S. Linn for comments on the manuscript. Supported by a Junior Fellowship from the Swiss National Foundation (A.E.), NIH grant GM31105 (J.R.), and a Mutagenesis Center grant from the National Institute of Environmental Health Sciences for the support of core facilities (P30ESO1896-12, B. Ames).

6 October 1995; accepted 13 November 1995

A *Drosophila* Homolog of the Yeast Origin Recognition Complex

Manfred Gossen,* Daniel T. S. Pak,* Stig K. Hansen, Jairaj K. Acharya, Michael R. Botchan†

Genes from *Drosophila melanogaster* have been identified that encode proteins homologous to Orc2p and Orc5p of the *Saccharomyces cerevisiae* origin recognition complex (ORC). The abundance of the *Drosophila* Orc2p homolog DmORC2 is developmentally regulated and is greatest during the earliest stages of embryogenesis, concomitant with the highest rate of DNA replication. Fractionation of embryo nuclear extracts revealed that DmORC2 is found in a tightly associated complex with five additional polypeptides, much like the yeast ORC. These studies will enable direct testing of the initiator-based model of replication in a metazoan.

DNA replication in higher eukaryotes is intricately regulated, both temporally and spatially, within each cell cycle and throughout development. The mechanisms underlying this regulation remain largely obscure, primarily because the sites and proteins involved in initiation have not been clearly identified. In the classic replicon model (*1*), a positive-acting factor (the initiator) acts at a specific DNA sequence (the replicator) to direct DNA synthesis to a nearby start site, the origin of replication. This concept has proven to be valid for prokaryotes as well as for eukaryotic DNA viruses (*2*). However, metazoan initiators that recognize chromosomal DNA have not been described. Moreover, the exact nature of the metazoan replicon is subject to controversy; start sites of DNA replication have been mapped to relatively short DNA sequences or to widespread initiation zones, depending on the origin region under investigation and on the techniques used (*3*).

The current state of knowledge concerning the initiation of chromosomal DNA replication in eukaryotes derives mainly from studies in *S. cerevisiae*, for which both an initiator and replicators have been identified. The ORC is a six-subunit assembly that binds in a site-specific, adenosine triphosphate (ATP)–dependent manner to autonomously replicating sequence (ARS) consensus sites, which define budding yeast origins of DNA replication (*4*). Despite the large number of replicator sites in a yeast chromosome and the sequence variations within these sites, it appears as if one vital initiator complex is central to the orchestration of the events that lead to DNA synthesis. The ORC influences the rate of firing at all active origins that have been examined (*5*) and also participates in transcriptional silencing at ARS elements of the yeast mating type loci (*6, 7*). Three ORC subunits (Orc2p, Orc5p, and Orc6p) have been described; they have been shown to be essential for viability and, in the case of Orc2p and Orc5p, for plasmid maintenance as well (*6, 8, 9*).

A genomic DNA sequence in the region of the *Drosophila* genome proximal to the *inositol polyphosphate-1-phosphatase* gene (*IPP*) showed homology to *S. cerevisiae ORC2* (*10*). We used the initially defined region of sequence homology to generate hybridization probes, and we identified complementary DNA (cDNA) clones in two independent early embryonic libraries (*11*). The sequences of these cDNAs predicted a protein that showed ~30% amino acid identity and ~56% homology to the COOH-terminus (residues 430 to 547) of *S. cerevisiae* Orc2p (Fig. 1B). Because the homology was less pronounced at the NH_2-termini of the two proteins, the average amino acid identity was 21% (37% homology).

Antibodies to this *Drosophila* Orc2p homolog, DmORC2 (*12*), were used to analyze protein extracts from *Drosophila* embryos col-

M. Gossen, D. T. S. Pak, M. R. Botchan, Division of Biochemistry and Molecular Biology, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA.
S. K. Hansen, Howard Hughes Medical Institute, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA.
J. K. Acharya, Howard Hughes Medical Institute, Departments of Biology and Neurosciences, University of California, San Diego, La Jolla, CA 92093–0649, USA.

*These authors contributed equally to this report.
†To whom correspondence should be addressed.

lected at various time points during development. DmORC2 was most abundant in embryos 0 to 4 hours old (Fig. 2) and constituted ~0.01% of total protein at this stage. Fractionation of embryos into nuclear and cytosolic extracts (*13*) and protein immunoblotting of equal amounts of protein from these extracts with antibodies to DmORC2 (anti-DmORC2) showed that DmORC2 was localized predominantly in the nucleus. Accordingly, we processed embryo nuclear extracts by conventional chromatography to determine whether DmORC2 was part of a multiprotein complex, as would be expected if the eukaryotic DNA replication initiation machinery is conserved.

Nuclear extracts (*13*) were first fractionated on a heparin-POROS (Perseptive Biosystems) matrix, and the elution of DmORC2 was followed by protein immunoblot analysis with affinity-purified anti-DmORC2 (*12*). The eluate that contained DmORC2 was pooled and further fractionated (Fig. 3A). About half of the DmORC2 separated on the Sephacryl-300 (Pharmacia) step into a high-molecular-mass fraction estimated to be >500 kD, whereas the remainder appeared to be in complexes of lower molecular mass. We continued to analyze the composition of the higher molecular mass complex. Ion-exchange chromatography with Mono-Q (Pharmacia) (Fig. 3A) showed that DmORC2 eluted in a sharp peak, which implied a homogeneous physical interaction with the matrix.

Immunoprecipitation of DmORC2 from the Mono-Q peak fractions and analysis of the immunoprecipitates by SDS–polyacrylamide gel electrophoresis (PAGE) revealed that DmORC2 was consistently associated with five other proteins (Fig 3B). This complex of six polypeptides was also observed after the complex was washed in buffer containing 1 M KCl or 1 M urea (*12*). The molecular mass of each polypeptide was inferred by the electrophoretic mobility of the protein bands relative to silver-stained size standards in several independent immunoprecipitation experiments (Fig. 3B). The sum of these molecular masses and the apparent stoichiometry of the subunits yielded a predicted molecular mass of 395 kD for the *Drosophila* complex; this value is close to the 413 kD estimated by similar means for the yeast complex.

The Mono-Q material was further fractionated on a Mono-S column, from which the DmORC2-containing peak eluate was analyzed by glycerol gradient centrifugation (Fig. 3C); the results again demonstrated that DmORC2 is contained in a complex. The sedimentation data revealed an apparent molecular mass of ~235 kD for the DmORC2 complex (comigrating with the catalase marker), as detected by protein immunoblotting with anti-DmORC2. This peak coincided with the peak of the five other *Drosophila* proteins, as judged by silver staining of the glycerol gradient fractions containing DmORC2 (Fig. 3C). Yeast ORC also has an apparent sedimentation coefficient close to that of catalase (*4*). Recombinant DmORC2 (*12*), in contrast, sedimented as a sharp peak at an apparent molecular mass of ~100 kD. We concluded from these data that the complex characterized here had properties that were similar to those of ORC purified from budding yeast with respect to the number of subunits, combined molecular mass, and sedimentation behavior. Thus, it was likely that the two complexes had homologous structures.

Given these similarities between the *Drosophila* and yeast ORCs, we anticipated that the DmORC2-associated proteins would have homology to other yeast ORC subunits. We cloned a gene with significant sequence homology to *S. cerevisiae ORC5* on the basis of sequence information released from the *Drosophila* Genome Project (*14*). Complementary DNAs of the putative *DmORC5*, isolated from the embryonic library that contained full-length *DmORC2* cDNAs (*11*), revealed an open reading frame (ORF) with

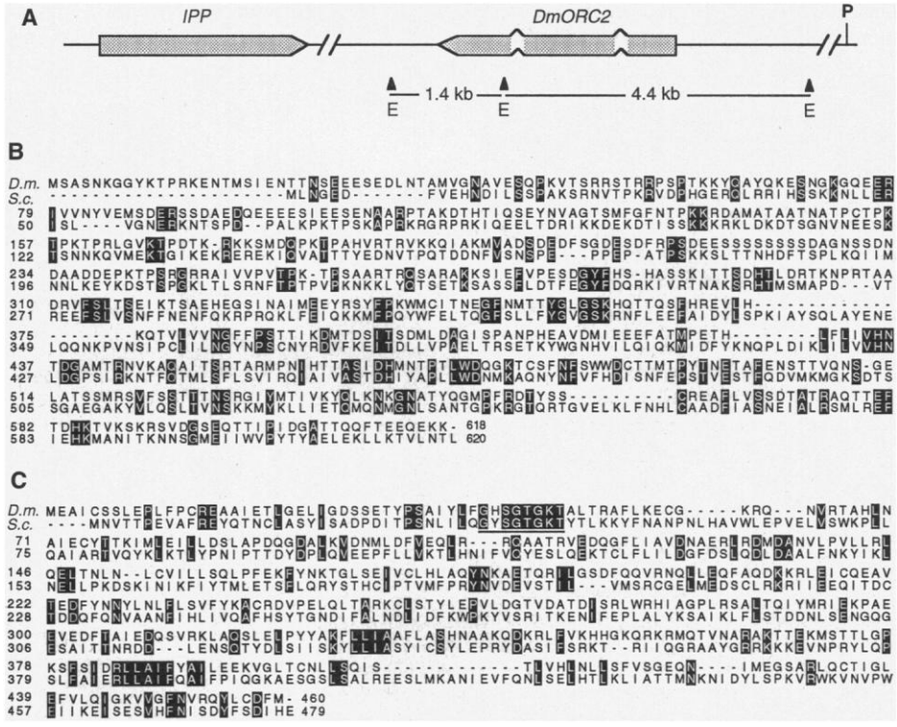

Fig. 1. *Drosophila* homologs of *S. cerevisiae* ORC proteins. (**A**) Schematic presentation of the genomic region of the *Drosophila DmORC2* gene. The ORFs of the *IPP* and *DmORC2* genes are indicated by arrowheads. Featured in the map are the Eco RI restriction sites (E) used for the initial subcloning of *DmORC2*, the position of the P-element insertion (P) in the particular *Drosophila* strain in which *DmORC2* was initially found, and the positions of the two introns (nonshaded areas) in the genomic *DmORC2* DNA. (**B**) The deduced amino acid sequence encoded by the *DmORC2* gene is shown in alignment (*22*) with *S. cerevisiae* Orc2p. Identical residues are highlighted. (**C**) The deduced amino acid sequence encoded by the *DmORC5* gene is shown in alignment with *S. cerevisiae* Orc5p. The putative nucleotide binding site is underlined. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

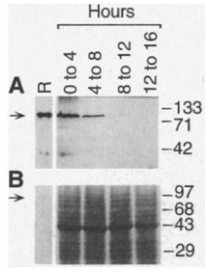

Fig. 2. Abundance of DmORC2 in early stages of *Drosophila* development. (**A**) Protein immunoblot analysis of total embryo extracts at various developmental stages. Collected embryos were subjected to Dounce homogenization in SDS sample buffer. Extracts were fractionated by SDS-PAGE and transferred to a nylon membrane. Protein was incubated with DmORC2-specific rabbit antiserum (*12*) and visualized by chemiluminescence with horseradish peroxidase–coupled protein A and ECL reagents (Amersham). (**B**) A Coomassie blue–stained gel of the same material used in (A) to verify that equivalent amounts of total protein were analyzed for DmORC2 at the different stages. Half the amount of cell extract was loaded per lane relative to (A). Recombinant DmORC2 (R) was used as a standard [10 ng in (A), 150 ng in (B)]. The positions and sizes (in kilodaltons) of the molecular mass markers are indicated at the right.

22% identity and 39% homology to yeast Orc5p (Fig. 1C). The predicted NH_2-terminus of DmORC5 contained a purine nucleotide binding site P-loop (*15*), as does yeast Orc5p (*8*). However, the utility of this putative ATP binding site in yeast is unclear (*8*). The in vitro translated product from the *DmORC5* cDNA comigrated in SDS-PAGE with the fourth largest subunit of the *Drosophila* ORC (Fig. 3B). The identity of this subunit with the predicted gene product of *DmORC5* was confirmed by peptide sequence analysis (*16*).

Taken together, our data suggest that this *Drosophila* complex is a functional homolog of the yeast initiator ORC. Indeed, *DmORC2* was capable of partially complementing the silencing defect in the *S. cerevisiae* temperature-sensitive *orc2-1* mutant, which demonstrated functional conservation between these two genes (*17*). Additionally, the distribution of DmORC2 during embryogenesis (Fig. 2) correlated with the observation that major waves of rapid cell division occur in embryos during the first 4 hours of development and then taper off (*18*). In these rapid nuclear cleavages, the requisite high rate of DNA synthesis is achieved, at least in part, by a frequency of detectable active origins that is 100 times the frequency seen in cultured *Drosophila* cell lines (*19*). Initiator proteins that determine origins of replication in *Drosophila* are therefore expected to be abundant during early development, whereas they may be much less prominent at later time points, when there are fewer active origins.

If DmORC proves to be a bona fide initiator, we would expect to find its cognate replicator sequence. The interaction between these two components in *Drosophila* may, however, be subject to temporal and spatial control during development. In view of this potential complexity, the accessibility of *Drosophila* extracts to biochemical analysis (*20*) should facilitate the identification of replicators in a metazoan and of the mechanisms that underlie initiation of DNA replication at these loci.

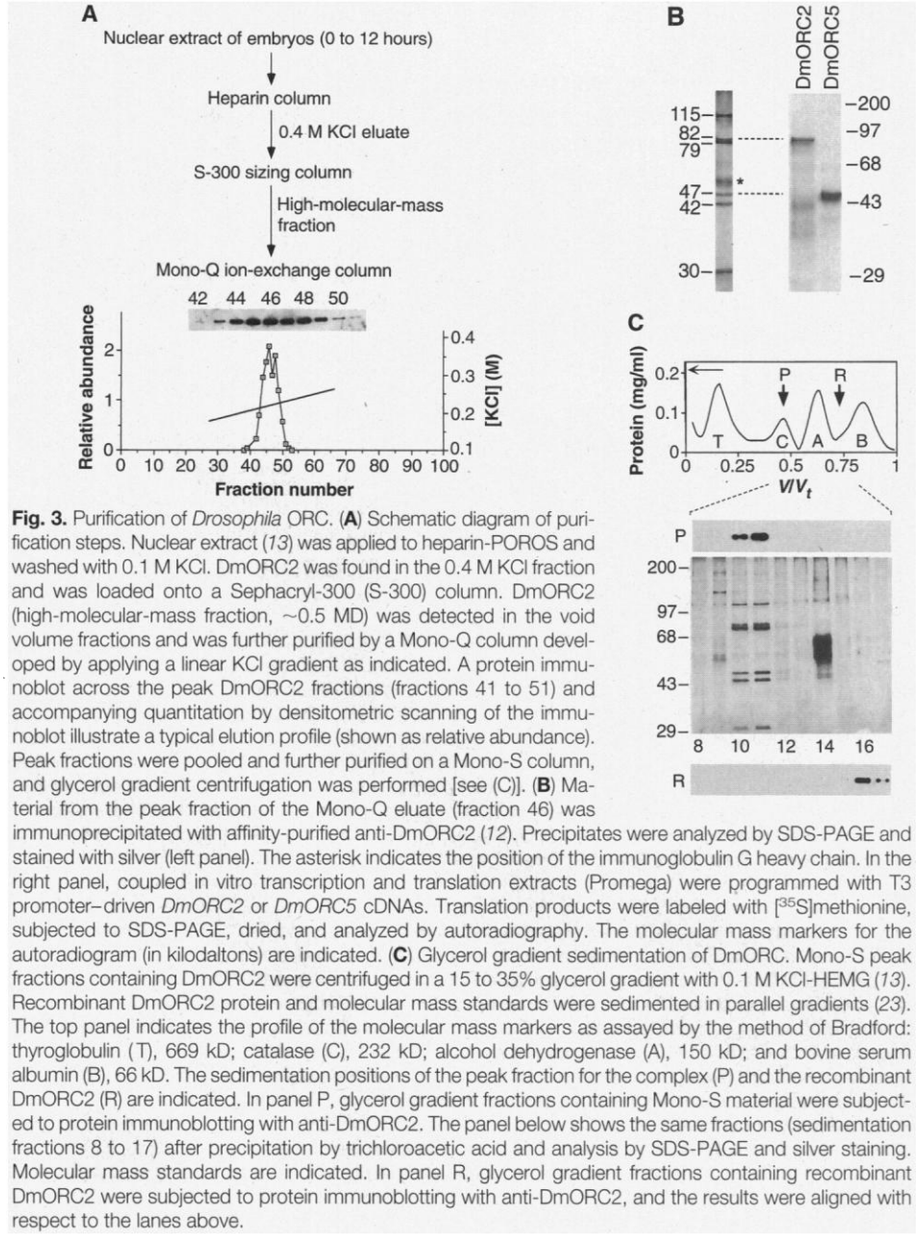

Fig. 3. Purification of *Drosophila* ORC. (**A**) Schematic diagram of purification steps. Nuclear extract (*13*) was applied to heparin-POROS and washed with 0.1 M KCl. DmORC2 was found in the 0.4 M KCl fraction and was loaded onto a Sephacryl-300 (S-300) column. DmORC2 (high-molecular-mass fraction, ~0.5 MD) was detected in the void volume fractions and was further purified by a Mono-Q column developed by applying a linear KCl gradient as indicated. A protein immunoblot across the peak DmORC2 fractions (fractions 41 to 51) and accompanying quantitation by densitometric scanning of the immunoblot illustrate a typical elution profile (shown as relative abundance). Peak fractions were pooled and further purified on a Mono-S column, and glycerol gradient centrifugation was performed [see (C)]. (**B**) Material from the peak fraction of the Mono-Q eluate (fraction 46) was immunoprecipitated with affinity-purified anti-DmORC2 (*12*). Precipitates were analyzed by SDS-PAGE and stained with silver (left panel). The asterisk indicates the position of the immunoglobulin G heavy chain. In the right panel, coupled in vitro transcription and translation extracts (Promega) were programmed with T3 promoter–driven *DmORC2* or *DmORC5* cDNAs. Translation products were labeled with [^{35}S]methionine, subjected to SDS-PAGE, dried, and analyzed by autoradiography. The molecular mass markers for the autoradiogram (in kilodaltons) are indicated. (**C**) Glycerol gradient sedimentation of DmORC. Mono-S peak fractions containing DmORC2 were centrifuged in a 15 to 35% glycerol gradient with 0.1 M KCl-HEMG (*13*). Recombinant DmORC2 protein and molecular mass standards were sedimented in parallel gradients (*23*). The top panel indicates the profile of the molecular mass markers as assayed by the method of Bradford: thyroglobulin (T), 669 kD; catalase (C), 232 kD; alcohol dehydrogenase (A), 150 kD; and bovine serum albumin (B), 66 kD. The sedimentation positions of the peak fraction for the complex (P) and the recombinant DmORC2 (R) are indicated. In panel P, glycerol gradient fractions containing Mono-S material were subjected to protein immunoblotting with anti-DmORC2. The panel below shows the same fractions (sedimentation fractions 8 to 17) after precipitation by trichloroacetic acid and analysis by SDS-PAGE and silver staining. Molecular mass standards are indicated. In panel R, glycerol gradient fractions containing recombinant DmORC2 were subjected to protein immunoblotting with anti-DmORC2, and the results were aligned with respect to the lanes above.

REFERENCES AND NOTES

1. F. Jacob, S. Brenner, F. Cuzin, *Cold Spring Harbor Symp. Quant. Biol.* **28**, 329 (1963).
2. A. Kornberg and T. A. Baker, *DNA Replication* (Freeman, New York, 1992).
3. J. L. Hamlin and P. A. Dijkwel, *Curr. Opin. Genet. Dev.* **5**, 153 (1995).
4. S. P. Bell and B. Stillman, *Nature* **357**, 128 (1992).
5. C. A. Fox, S. Loo, A. Dillin, J. Rine, *Genes Dev.* **9**, 911 (1995); C. Liang, M. Weinrich, B. Stillman, *Cell* **81**, 667 (1995).
6. M. Foss, F. J. McNally, P. Laurenson, J. Rine, *Science* **262**, 1838 (1993); S. P. Bell, R. Kobayashi, B. Stillman, *ibid.*, p. 1844.
7. G. Micklem, A. Rowley, J. Harwood, K. Nasmyth, J. F. X. Diffley, *Nature* **366**, 87 (1993).
8. S. Loo *et al.*, *Mol. Biol. Cell* **6**, 741 (1995).
9. J. J. Li and I. Herskowitz, *Science* **262**, 1870 (1993).
10. The *Drosophila* genomic DNA that potentially encodes a protein with sequence similarity to yeast Orc2p was identified in the course of analyzing the nearby *IPP* gene. A P-element insertion in close proximity to the *IPP* gene was rescued, and the genomic DNA was further subcloned as Eco RI fragments. The homology to yeast Orc2p was identified by sequencing the insert boundaries of two of these subclones, which were later identified as sequences flanking the Eco RI site indicated within the *DmORC2* ORF (Fig. 1A).
11. A plasmid cDNA library from 4- to 8-hour *Drosophila* embryos [N. H. Brown and F. C. Kafatos, *J. Mol. Biol.* **203**, 425 (1988)] was transformed into *Escherichia coli* SCS-1 (Stratagene); 1.4×10^6 transformants were obtained and were transferred at high density to nylon membranes. These membranes were screened [D. Hanahan and M. Meselson, *Recombinant DNA Methodology* (Academic Press, London, 1989), pp. 267–275] with ^{32}P-labeled DNA fragments from the *Drosophila* genomic subclones (*10*) that encompassed the sequence homology to the yeast *ORC2* gene. About 80 positive clones could be identified in the initial hybridization, and eight were isolated. Restriction enzyme analysis indicated that they contained a common cDNA sequence. The nucleotide sequence of the longest cDNA isolated (clone 2-2) revealed an ORF (Fig. 1B). In parallel, a λgt10 cDNA library from 3- to 12-hour *Drosophila* embryos, which contained 5×10^5 independent clones, was used to infect BNN102 hosts, and 3×10^5 plaques were screened with the same hybridization probes as described above. Thirty clones remained after three rounds of plaque purification, of which 10 were characterized. All phage clones contained sequences that matched the ORF of clone 2-2.
12. *DmORC2* was cloned into the T7 expression vector RSET-A (Invitrogen) fused to an epitope encoding six histidines. The fusion construct was transformed into *E. coli* BL21 LysS (Novagen). Expression of His-tagged DmORC2 was induced with isopropyl-β-D-thiogalactopyranoside, and the protein was purified by nickel-chelate chromatography according to standard procedures. For expression of the protein in Sf9 cells, *DmORC2* was inserted into a BaculoGold vector (Pharmingen) and expressed with a polyoma middle-T antigen epitope tag. The baculovirus-expressed DmORC2 was purified by immunoaffinity chromatography with the use of protein G–Sepharose (Pharmacia) conjugated to monoclonal antibodies against the polyoma epitope tag.

Polyclonal rabbit antibodies were obtained after three or four immunizations with both *E. coli*– and baculovirus-expressed DmORC2. These polyclonal antibodies were affinity-purified (*21*) by incubation with baculovirus-produced DmORC2 protein coupled to Reactigel beads (Pierce). The eluted antibodies were bound to protein A–Sepharose (Pharmacia) and chemically cross-linked to this support with dimethylpimelimidate (*21*). For immunoprecipitation, 1 μl of these beads was added directly to the respective chromatographic or glycerol gradient fraction, adjusted to 100 mM KCl, incubated for 2 hours at 4°C, and washed six times with 0.1 M KCl–HEMG [25 mM Hepes (pH 7.6), 0.5 mM EDTA, 12.5 mM $MgCl_2$, and 10% glycerol] (*14*). The recovered material was resuspended directly in SDS sample buffer and analyzed by SDS-PAGE.

13. U. Heberlein and R. Tjian, *Nature* **331**, 410 (1988).
14. *Drosophila* Genome Center, Lawrence Berkeley Laboratory, Berkeley, CA; GenBank accession number L39626.
15. E. V. Koonin, *Nucleic Acids Res.* **21**, 2541 (1993).
16. S. K. Hansen, D. T. S. Pak, M. Gossen, S. Zhou, M. R. Botchan, unpublished data.
17. A. E. Ehrenhofer-Murray, M. Gossen, D. T. S. Pak, M. R. Botchan, J. Rine, *Science* **270**, 1671 (1995).
18. V. E. Foe, G. M. Odell, B. A. Edgar, in *The Development of* Drosophila melanogaster, M. Bate and A. M. Arias, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), vol. 1, pp. 149–300.
19. A. B. Blumenthal, H. J. Kriegstein, D. S. Hogness, *Cold Spring Harbor Symp. Quant. Biol.* **38**, 205 (1974); S. L. McKnight and O. L. Miller, *Cell* **12**, 795 (1977); V. A. Zakian, *J. Mol. Biol.* **108**, 305 (1976).
20. R. T. Kamakaka, P. D. Kaufman, B. Stillman, P. G. Mitsis, J. T. Kadonaga, *Mol. Cell. Biol.* **14**, 5114 (1994); C. S. Chiang, P. G. Mitsis, I. R. Lehman, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9105 (1993).
21. E. Harlow and D. Lane, Eds., *Antibodies* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988).
22. Alignments were performed with the ClustalW program [J. D. Thompson, D. G. Higgins, T. J. Gibson, *Nucleic Acids Res.* **22**, 4673 (1994)].
23. Fraction number variation among different glycerol gradients (23 fractions for the molecular mass standards, 20 fractions for recombinant DmORC2, and 21 fractions for the complex) was corrected by normalization of each fraction to V/V_t (the ratio of cumulative eluted volume to total gradient volume). The leftmost and rightmost lanes of each protein immunoblot and silver stain correspond to fractions eluted at 36% and 79% of the total volume, respectively.
24. We thank C. Zuker for providing the *DmORC2* genomic clone, D. Rio for bringing the initial finding of the Zuker lab to our attention, M. Levine (University of California, San Diego) for the plasmid cDNA library, T. Kornberg for the λgt10 cDNA library, D. Rio and R. Tjian for encouragement and technical advice, and A. Ho for help. Supported by a pilot project grant from the National Institute of Environmental Health Sciences Mutagenesis Center (ES-01896), National Cancer Institute grant CA30490, and a Human Frontier Science Program Long-Term Research Fellowship (M.G.). Sequences of the *DmORC2* and *DmORC5* cDNAs have been deposited in GenBank (accession numbers pending).

6 October 1995; accepted 13 November 1995

Early-Onset Epilepsy and Postnatal Lethality Associated with an Editing-Deficient *GluR-B* Allele in Mice

Rossella Brusa, Frank Zimmermann, Duk-Su Koh, Dirk Feldmeyer, Peter Gass, Peter H. Seeburg, Rolf Sprengel

The arginine residue at position 586 of the GluR-B subunit renders heteromeric α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)–sensitive glutamate receptor channels impermeable to calcium. The codon for this arginine is introduced at the precursor messenger RNA (pre-mRNA) stage by site-selective adenosine editing of a glutamine codon. Heterozygous mice engineered by gene targeting to harbor an editing-incompetent *GluR-B* allele synthesized unedited GluR-B subunits and, in principal neurons and interneurons, expressed AMPA receptors with increased calcium permeability. These mice developed seizures and died by 3 weeks of age, showing that GluR-B pre-mRNA editing is essential for brain function.

Glutamate receptors sensitive to AMPA are ligand-activated cation channels that mediate the fast component of excitatory postsynaptic currents in central neurons (*1*). These channels are assembled from four related subunits (GluR-A to GluR-D, or GluR1 to GluR4) (*2*), with the GluR-B subunit rendering the channel almost impermeable to Ca^{2+} (*3*). The molecular determinant for this dominant property of GluR-B was traced to the arginine (R) residue at position 586 of the mature subunit, which lies within the pore-forming segment M2 (*4*). This arginine is not gene encoded (*5*) but is posttranscriptionally introduced into GluR-B pre-mRNA (*5*, *6*) by site-selective adenosine deamination, which leads to the change of a CAA glutamine (Q) codon to a CIG codon for arginine in >99% of mRNA molecules (*5*–*7*). Termed Q/R site editing, this nuclear process depends on a double-stranded RNA structure (*6*) formed in the pre-mRNA by the editing site in exon 11 and the editing site complementary sequence (ECS) in intron 11 (*8*). To investigate in an animal model the relevance of this process for central nervous system (CNS) physiology, we targeted intron 11 of the *GluR-B* gene in mouse embryonic stem (ES) cells (*9*) for replacement of the ECS element (*10*) by *loxP* (*11*, *12*) (Fig. 1), and then injected correctly engineered cells into C57BL/6 blastocysts. One of several resultant chimeric animals showed vertical transmission of the *GluR-B*$^{\Delta ECS}$ allele in a Mendelian fashion (*10*), indicating that the allele did not adversely affect embryonic development.

In brains of *GluR-B*$^{+/\Delta ECS}$ mice, the *GluR-B*$^{\Delta ECS}$ allele was expressed with its transcripts remaining unedited at the Q/R site, demonstrated by analysis of allele-specific reverse transcription–polymerase chain reaction (RT-PCR) products (*13*) of partially spliced GluR-B pre-mRNA (Fig. 2A). The sequence and hybridization analysis of RT-PCR products revealed that pre-mRNA derived from the wild-type allele was edited to the expected extent of 83% (*6*, *13*), whereas pre-mRNA from the *GluR-B*$^{\Delta ECS}$ allele, in which the ECS element was replaced by *loxP*, was not edited at the Q/R site. These data showed that the ECS element is indispensable for Q/R site editing in vivo, as previously established for in vitro editing (*6*, *7*). The RT-PCR analysis further indicated that *GluR-B*$^{\Delta ECS}$ pre-mRNA sequences were amplified more efficiently than *GluR-B*$^{+}$ pre-mRNA (Fig. 2A). Quantification with primers that amplify DNA fragments of identical size for both allelic pre-mRNAs (*13*) revealed that premature transcripts of the *GluR-B*$^{\Delta ECS}$ allele are enriched approximately fivefold in the nucleus as compared with premature transcripts of the *GluR-B*$^{+}$ allele. The nuclear accumulation of *GluR-B*$^{\Delta ECS}$ pre-mRNA was attributable to a reduced splicing efficiency of the sequence-modified intron 11, because ribonuclease (RNase) protection with a suitable intron probe (*14*) revealed increased amounts of the *loxP*-containing intron 11 relative to the unmodified intron (Fig. 2B). Consequently, the amounts of cytoplasmic mRNA corresponding to the two alleles were imbalanced, with mature cytoplasmic transcripts unedited at the Q/R site constituting only 25 ± 3% (mean ± SEM, $n = 8$), rather than the theoretically expected 50%, of the GluR-B mRNA population. This situation reflects an overall decrease in GluR-B mRNA abundance, and, indeed, a reduction of ~30% in the amount of GluR-B mRNA was demonstrated by densitometric analysis of Northern

R. Brusa, F. Zimmermann, P. H. Seeburg, R. Sprengel, Laboratory of Molecular Neuroendocrinology, Center for Molecular Biology (ZMBH), University of Heidelberg, Im Neuenheimer Feld 282, D-69120 Heidelberg, Germany.
D.-S. Koh, D. Feldmeyer, B. Sakmann, Max-Planck-Institut für Medizinische Forschung, Abteilung Zellphysiologie, Jahnstrasse 29, D-69120 Heidelberg, Germany.
P. Gass, Institut für Neuropathologie, University of Heidelberg, Im Neuenheimer Feld 220, D-69120 Heidelberg, Germany.