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Embryonic Lethality of Mutant Mice Deficient in the p116 Gene[1]

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We report a lethal phenotype of mouse embryo with a disruption in the gene encoding p116, a subunit of the translation initiation factor, eIF3. The amino acid sequence of mouse p116, as deduced from the cDNA, shows high homology (97%) with human p116, and contains the conserved RNA binding sites, RNP1 and RNP2. The p116 mRNA is ubiquitously expressed in various organs, suggesting a house-keeping function of the p116 protein. To obtain genetic evidence for the essential role of the p116 protein in mouse cells, we constructed mice with a disruption in the p116 gene. Heterozygous $p116^{+/-}$ mice were intercrossed, and the genotypes of the offspring were determined. The results indicated no $p116^{-/-}$ pups among 84 neonates. Also, there were no $p116^{-/-}$ embryos 13.5 days postcoitum (d.p.c.). Among 77 embryos, there was only one $p116^{-/-}$ embryo at the blastocyst stage (3.5 d.p.c.). These results indicate that p116 plays an essential role in the early stages of mouse development.

Key words: eIF3, embryonic lethal, gene trapping, knockout mouse, p116, Prt1, translation initiation.

The initiation of translation in eukaryotes is promoted by soluble proteins called eukaryotic initiation factors (eIFs) (*1–4*). eIF3, which was originally purified from an extract of rabbit reticulocytes and HeLa cells, is the largest of these factors and is a multiple protein complex comprising at least 9 subunits, p170, p116, p166, p48, p47, p44, p40, p36, and p35 (*5–9*). eIF3 plays a central role in the translation initiation pathway. eIF3 has also been purified from yeast (*10, 11*). cDNA cloning of human p116 revealed that the amino acid sequence shares 30% homology with Prt1, a yeast homologue of mammalian p116 (*10, 12*). Purified Prt1 stimulates the initiation of translation *in vitro* (*10*). Protein synthesis is inhibited in a temperature-sensitive mutant of the prt1 gene, which encodes Prt1, due to an inability to form the translation initiation complex (*13, 14*). Thus, Prt1 is essential for the initiation of translation in yeast. These results suggest that p116 is necessary for the initiation of translation in mammalian cells; however, there is no genetic evidence that this is the case.

The establishment of ES cells with a disruption in a particular gene by insertion of a trap vector into chromosomal DNA, followed by construction of chimeric mice by introducing the ES cells into blastocysts, and the generation of mice with disrupted genes, is useful for examining the functions of the protein encoded by the disrupted gene (*15*). In the present study, we established an ES cell line whose p116 gene was disrupted by the insertion of a trap vector, and constructed heterozygous $p116^{+/-}$ mice. Intercrossing the heterozygous $p116^{+/-}$ mice revealed that the protein encoded by the p116 gene is essential for the early stages of mouse development.

MATERIALS AND METHODS

Establishment of Mutant ES Cell Lines by the Gene-Trapping Method—The gene-trapping method was performed as described by Baker *et al.* (*16*). The trap vector, pGT1.8Zin, was introduced into ES R1 cells (provided by Dr. Hisato Kondo, Osaka University) by electroporation. The cells were cultured at 37°C in ES culture medium (Dulbecco's modified Eagle's medium; DMEM; Invitrogen, Carlsbad, CA) containing 0.0007% β-mercaptoethanol (Nacalai Tesque, Japan), 100 μmol/liter non-essential amino acids (Invitrogen), 1 mg/ml nucleosides (Sigma Chemical, St. Louis, MO), 10^3 U/ml ESGRO (Invitrogen), and 20% fetal bovine serum (Equitech-Bio, Kerrville, TX) supplemented with 250 μg/ml neomycin under a 5% CO_2 atmosphere. Colonies were further screened by β-galactosidase assay with X-gal staining. ES cells were fixed with glutaraldehyde, permeabilized by deoxycholic acid, and incubated with X-gal overnight at 37°C. β-Galactosidase–positive cell lines were selected and used for further analyses.

5′-Race Method for Amplification of cDNA—RNA was prepared from ES cells by the thiocyanateguanidine method. A primer (5′-AGG GTT TTC CCA GTC ACG ACG ACG-3′) corresponding to the downstream region of the lacZ gene in the trapped gene was used for cDNA synthesis with reverse transcriptase and RNA prepared from ES cells. PolyC was added to the 5′ end of the cDNA by the terminal deoxynucleotidyl transferase reaction. The polymerase chain reaction (PCR) method was performed with primers for the lacZ gene (5′-TGC TCT GTC AGG TAC CTG TTG G-

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3′) and polyC (5′-GGG IIG GGI GGG IIG-3′).

Cloning of Full Length Mouse p116 cDNA—Full length mouse p116 cDNA was amplified by the PCR method with DNA from a cDNA library of mouse ES cells. Three pairs of primers (A, B, and C; A 5′-CGA CGT GAG CGA GGA AGA ACT AC-3′ and 5′-TTC CTC ACT CGG ATC TTC TGT C-3′; B 5′-AAT CAT CTG GGA CAT CCT CAC GG-3′ and 5′-CGC TCG TTC TTC TGC TTC ATG TAG-3′; C 5′-GAT GTT TGA CAA GCA GCA AGC C-3′, and 5′-CAA GTC CGT GAA TGG AAA CCC-3′) were designed from a sequence obtained by the 5′-Race method of mouse p116 cDNA described above and human p116 cDNA. These primers cover the region upstream of the translation initiation site and the region downstream of the translation termination site of the p116 mRNA.

Northern Blot Hybridization—Total RNA was extracted from ES cells by the thiocyanateguanidine method, and poly $(A)^+$ RNA was further purified. After electrophoresis, poly $(A)^+$ RNA (1.5 μg/lane) was blotted onto a nylon membrane, and fixed by heat treatment at 80°C for 2 h. Prehybridization was performed at 65°C for 15 min, and the hybridization reaction was performed at 65°C for 2.5 h. The cDNA sequence corresponding to the 5′ upstream region of the trap vector insertion site was radio-labeled with ^{32}P, and used as a probe. The membrane was washed with 2× SSC at 65°C for 15 min, and then with 0.5× SSC at 65°C for 30 min. Autoradiography was performed with an imaging plate and analyzed with a BAS2000 (Fuji Photo Film, Tokyo).

Construction of p116 Gene Knockout Mice Using the Aggregation Chimera Method—Chimeric mice were established using ES cell aggregation as described by Kondoh *et al.* (*17*). BDF-1 mouse embryos at the morula stage were co-cultured with GT529 cells, developed into blastocysts, and transplanted into the uteri of female ICR mice. Among the derived pups, chimeric mice having agouti fur color were further crossed with the wild type C57BL/6J mice, and heterozygous $p116^{+/-}$ mice were obtained.

Determination of the Genotypes of Pups, Nonviable Embryos, and Blastocysts—Heterozygous $p116^{+/-}$ mice were intercrossed, and chromosomal DNA was extracted from the resulting pups, neonatal pups, and embryos at the blastocyst stage. PCR products were analyzed by agarose gel electrophoresis, and the genotypes were determined. The

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mouse    1 MQDAENVAVPEAAEERAEPARQQPASESPPTD---EAAGSGGSEVGQTEDAEE---DAEAGPEPEVRAK  63
human    1 MQDAENVAVPEAAEERAEPGQQQPAAEPPPAEGLLRPAGPGAPEAAGTEASSEEVGIAEAGPEPEVRTE  69
           ******************* .****.* **..     ** *  *   ** . *    **********.

mouse   64 PAAQSEEETATSPAASPTPQSAERSP--SQEPSAPGKAEAVGEQARGHPS-----AGAEEEGGSDGSAAEAEPRALENGE 136
human   70 PAA--EAEAASGPSESPSPPAAEELPGSHAEPPVPAQGEAPGEQARDERSDSRAQAVSEDAGGNEGRAAEAEPRALENGD 147
           ***  * *.*. *. **.* .**  *    **  * . ** *****   *     * .*. ** .* ************.

                                                  RNP1
mouse  137 ADEPSFSDPEDFVDDVSEEELLGDVLKDRPQEADGIDSVIVVDNVFQVGPDRLEKLKNVIHKIFSKFGKIINDYYPEEDG 216
human  148 ADEPSFSDPEDFVDDVSEEELLGDVLKDRPQEADGIDSVIVVDNVFQVGPDRLEKLKNVIHKIFSKFGKITNDFYPEEDG 227
           ************************************************************************ **.******

             RNP2
mouse  217 KTKGYIFLEYASPAHAVDAVKNADGYKLDKQHTFRVNLFTDFDKYMTISDEWDIPEKQPFKDLGNLRYWLEEAECRDQYS 296
human  228 KTKGYIFLEYASPAHAVDAVKNADGYKLDKQHTFRVNLFTDFDKYMTISDEWDIPEKQPFKDLGNLRYWLEEAECRDQYS 307
           ********************************************************************************

mouse  297 VIFESGDRTSIFWNDVKDPVSIEERARWTETYVRWSPKGTYLATFHQRGIALWGGDKFKQIQRFSHQGVQLIDFSPCERY 376
human  308 VIFESGDRTSIFWNDVKDPVSIEERARWTETYVRWSPKGTYLATFHQRGIALWGGEKFKQIQRFSHQGVQLIDFSPCERY 387
           ****************************************************.***********************

mouse  377 LVTFSPLMDTQDDPQAIIIWDILTGHKKRGFHCESSAHWPIFKWSHDGKFFARMTLDTLSIYETPSMGLLDKKSLKISGI 456
human  388 LVTFSPLMDTQDDPQAIIIWDILTGHKKRGFHCESSAHWPIFKWSHDGKFFARMTLDTLSIYETPSMGLLDKKSLKISGI 467
           ********************************************************************************

mouse  457 KDFSWSPGGNIIAFWVPEDKDIPARVTLMQLPTRQEIRVRNLFNVVDCKLHWQKNGDYLCVKVDRTPKGTQGVVTNFEIF 536
human  468 KDFSWSPGGNIIAFWVPEDKDIPARVTLMQLPTRQEIRVRNLFNVVDCKLHWQKNGDYLCVKVDRTPKGTQGVVTNFEIF 547
           ********************************************************************************

mouse  537 RMREKQVPVDVVEMKETIIAFAWEPNGSKFAVLHGEAPRISVSFYHVKSNGKIELIKMFDKQQANTIFWSPQGQFVVLAG 616
human  548 RMREKQVPVDVVEMKETIIAFAWEPNGSKFAVLHGEAPRISVSFYHVKNNGKIELIKMFDKQQANTIFWSPQGQFVVLAG 627
           ********************************************** ******************************

mouse  617 LRSMNGALAFVDTSDCTVMNIAEHYMASDVEWDPTGRYVVTSVSWWSHKVDNAYWLWTFQGRLLQKNNKDRFCQLLWRPR 696
human  628 LRSMNGALAFVDTSDCTVMNIAEHYMASDVEWDPTGRYVVTSVSWWSHKVDNAYWLWTFQGRLLQKNNKDRFCQLLWRPR 707
           ********************************************************************************

mouse  697 PPTLLSQDQIKQIKKDLKKYSKIFEQKDRLSQSKASKELVERRRTMMEDFRQYRKMAQELYMKQKNERLELRGGVDTDEL 776
human  708 PPTLLSQEQIKQIKKDLKKYSKIFEQKDRLSQSKASKELVERRRTMMEDFRKYRKMAQELYMEQKNERLELRGGVDTDEL 787
           *******.****************************************.********** *****************

mouse  777 DSNVDDWEEETIEFFVTEEVIPLGSQE 803
human  788 DSNVDDWEEETIEFFVTEEIIPLGNQE 814
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Fig. 1. **Comparison of the amino acid sequences of mouse and human p116.** Asterisks indicate identical amino acids in mouse (upper) and human p116 (lower). Dots indicate conservative substitutions between mouse and human p116. Frames indicate RNA binding motifs that are highly conserved in eukaryotic p116 proteins and their homologues. Residue numbers are shown to the right side of the alignment. Gaps were introduced to maximize homology.

primers used for DNA from the tails of pups and whole bodies of neonatal pups were 5′-AGG ATT TCG TAG ACG ACG TGA GCG-3′ and 5′-AGT CAA TCC CGT CCG CTT CCT GAG G-3′ for detection of the wild type allele of the p116 gene, and 5′-GAT ACG TTA CGT TGG TGT AGA TGG GC-3′ and 5′-CTA CCA TGA AAA TTC AGC GCC C-3′ for detection of the mutant allele. DNA from blastocysts was analyzed by the PCR method using 5′-AGG ATT TCG TAG ACG ACG TGA GCG-3′ and 5′-AAC GTC CTC AAG GCT GGA G-3′ for the wild type allele of the p116 gene, and 5′-TTC TGC CGA GAA AGT ATC CA-3′ and 5′-GTC AAG AAG GCG ATA GAA GG-3′ for detection of the neomycin-resistant gene. To prepare blastocysts, heterozygous p116$^{+/-}$ mice were mated, and 2- or 4-cell embryos were isolated from the oviducts of the female mice 1.5 days after mating. The fertilized eggs were washed with M2 medium, and cultured in M16 medium at 37°C under a 5% CO_2 atmosphere. Culture was continued for 2 days, and blastocysts were obtained.

RESULTS AND DISCUSSION

Isolation of Mouse p116 cDNA—We established several ES cell lines whose genes were randomly disrupted by insertion of a trap vector, pZT1.8Zin. Among them, one cell line (GT529) was successfully transferred to a germ line. We characterized the disrupted gene in GT529. First, the total RNA was extracted from GT529, and the cDNA of the trapped mRNA was isolated by the 5′ Race method. Next, the cDNA corresponding to the 3′ downstream region of this mRNA was isolated. Sequence analysis of the whole cDNA indicated that the amino acid sequence of the protein encoded by the open reading frame in this mRNA had 93% homology with human p116 (Fig. 1). Furthermore, there were two RNA binding motifs, RNP1 and RNP2, which are highly conserved in eukaryotic p116 proteins (Fig. 1). These results indicate that the p116 gene was trapped in GT529 cells.

Northern blot analysis was performed with poly (A)$^+$ RNA prepared from various organs of C57BL/6J mice. The results indicated that the p116 mRNA was expressed ubiquitously in kidney, liver, lung, thymus, and brain (Fig. 2), suggesting that the p116 protein has a role as a housekeeping protein.

Embryonic Lethality of Homozygous p116$^{-/-}$—We generated chimeric mice by the aggregation method with the GT529. The chimeric mice were crossed with wild type mice, and heterozygous p116$^{+/-}$ mice were obtained. The PCR method was used to determine the genotypes of the offspring (Fig. 3a). There was no apparent difference between the heterozygous p116$^{+/-}$ mice and wild type mice in terms of their growth and appearance. Heterozygous p116$^{+/-}$ mice were intercrossed and the genotypes of the neonatal mice were determined using the PCR method. There were no homozygous p116$^{-/-}$ pups among the 84 offspring (Table I). To determine the stage at which the homozygous p116$^{-/-}$ animals die, embryos from heterozygous matings were genotyped. At 13.5 d.p.c., there were no homozygous p116$^{-/-}$ embryos. At 3.5 d.p.c., there was only

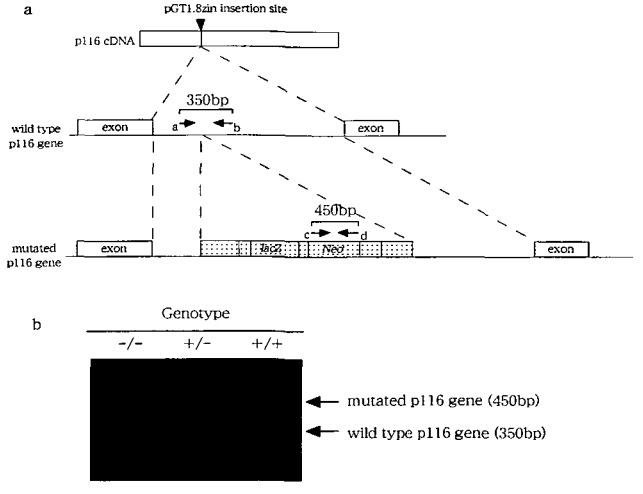

Fig. 3. **Genotyping of pups from the intercross of heterozygotes.** a: Diagram showing the insertion site of the trap vector in the knockout allele. Arrows indicate positions of primers. The sizes of the PCR products are shown. b: Genotyping of the blastocysts from the intercross of heterozygotes by the PCR method.

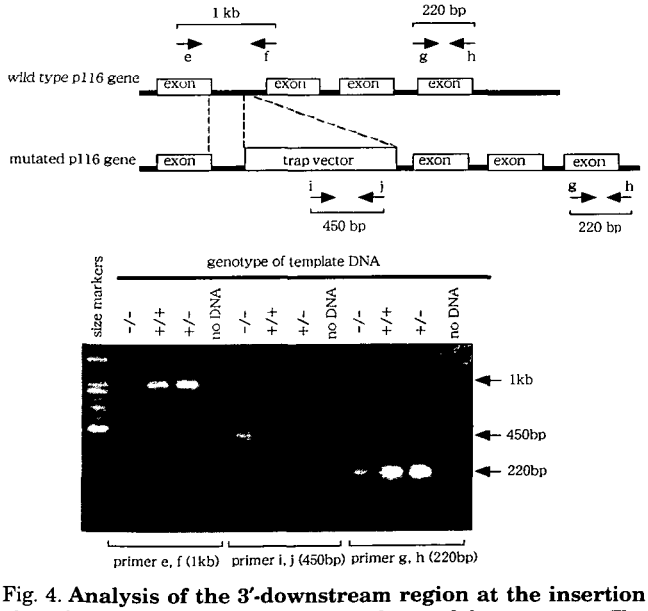

Fig. 4. **Analysis of the 3′-downstream region at the insertion site of the trap vector.** a: Diagram of part of the p116 gene. The insertion site of the trap vector in the knockout allele is shown. Arrows indicate positions of the primers used for the PCR method. The sizes of PCR products are shown. b: PCR analysis of blastocysts from the intercross of heterozygotes. Homozygous p116$^{-/-}$ (–/–), heterozygous p116$^{+/-}$ (+/–), and wild type (+/+) are shown.

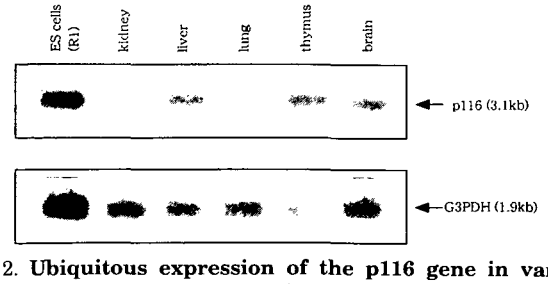

Fig. 2. **Ubiquitous expression of the p116 gene in various mouse organs.** p116 gene expression was assayed by Northern blot hybridization in ES cells, kidney, liver, lung, thymus, and brain.

TABLE I. **Homozygous p116$^{-/-}$ mice are embryonic lethal.**

Age	Genotype			Total
	+/+	+/–	–/–	
3.5 d.p.c. (blastocyst)	19	57	1	77
13.5 d.p.c.	2	13	0	15
4 weeks	29	55	0	84

Genotypes of offspring obtained by intercross of heterozygotes was determined by the PCR method. Homozygous p116$^{-/-}$ (–/–), heterozygous p116$^{+/-}$ (+/–), and wild type (+/+) are shown.

one homozygous p116$^{-/-}$ embryo among 77 embryos. Thus, we concluded that homozygous p116$^{-/-}$ is embryonic lethal, and that most embryos die before the blastocyst stage.

The ratio of wild type to heterozygous progeny was 29:55 at 4 weeks, consistent with the expected ratio of 1:2. Thus, both sperm and oocytes with the inactive allele are derived from productive gametogenesis and maintain their viability until fertilization. This result indicates that expression of the p116 gene is not required for viability of sperm or oocytes until fertilization. To confirm this, male or female heterozygous p116$^{+/-}$ mice were crossed with wild type mice, and the offspring genotypes were determined. In both cases, the ratio of heterozygous p116$^{+/-}$ mice to wild type was approximately 1:1 (data not shown), indicating that sperm and oocytes with the disrupted p116 gene have normal fertility. This indicates that the homozygous p116$^{-/-}$ mice died sometime between fertilization and the blastocyst stage. The reason for the production of functionally normal spermatozoa and oocytes by p116$^{+/-}$ mice is probably due to the active p116 protein derived from stem cells. However, we can not exclude the possibility that another protein compensates for the function of the p116 protein.

Analysis of the Genome Structure in the 3′ Downstream Region from the Disrupted p116 Gene—Disruption of genes by a trap vector can introduce a large deletion in the 3′-downstream region adjacent to the insertion site of the trap vector (*18*). This raises the possibility that the observed phenotype of embryonic lethality of the gene knockout mice might not be due exclusively to disruption of the p116 gene, and that deletion of other genes located in the 3′-downstream region of the p116 gene may also be responsible for the phenotype. To test this, we analyzed the structure of the 3′-downstream region of the p116 gene in the homozygous p116$^{-/-}$ embryo that developed to the blastocyst stage (Table I and Fig. 3b).

The PCR method was performed using primers located in the 3′ downstream region at the insertion site of the trap vector (Fig. 4). A 220-bp DNA fragment was amplified with the g and h primer set on chromosomal DNA from both wild type and the homozygous p116$^{-/-}$ embryos, indicating that the sequence was intact in the genome of the homozygous p116$^{-/-}$ embryo. Therefore, we conclude that no large deletion of chromosomal DNA adjacent to the insertion site of the trap vector occurred. Thus, disruption of the p116 gene alone is responsible for the embryonic lethal phenotype. In this study, we analyzed the structure of the downstream region of the p116 gene in the mutated allele by the PCR method. Further analyses, such as FISH, restriction mapping, PFGE, or Southern blotting, will be needed to reach an absolute conclusion.

In this study, we cloned p116 cDNA and established gene knockout mice. The results show that p116 is essential in the early stages of mouse development.

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REFERENCES

1. Merrick, W.C. and Hershey J.W.B. (1996) The pathway and mechanism of eukaryotic protein synthesis in *Translational Control* (Hershey, J.W.B., Mathews, M.B., and Sonenberg, N., eds.) pp. 31–69, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Sachs, A.B., Sarnow, P., and Hentze, M.W. (1997) Starting at the beginning, middle, and end: translation initiation in eukaryotes. *Cell* **89**, 831–838
3. Gray, N.K. and Wickens, M. (1998) Control of translation initiation in animals. *Annu. Rev. Cell Dev. Biol.* **14**, 399–458
4. Preiss, T. and Hentze, M.W. (1999) From factors to mechanisms: translation and translational control in eukaryotes. *Curr. Opin. Genet. Dev.* **9**, 515–521
5. Hershey, J.W., Asano, K., Naranda, T., Vornlocher, H.P., Hanachi, P., and Merrick, W.C. (1996) Conservation and diversity in the structure of translation initiation factor EIF3 from humans and yeast. *Biochimie* **78**, 903–907
6. Benne, R. and Hershey J.W. (1976) Purification and characterization of initiation factor IF-E3 from rabbit reticulocytes. *Proc. Natl. Acad. Sci. USA* **73**, 3005–3009
7. Safer, B., Adams, S.L., Kemper, W.M., Berry, K.W., Lloyd, M., and Merrick, W.C. (1976) Purification and characterization of two initiation factors required for maximal activity of a highly fractionated globin mRNA translation system. *Proc. Natl. Acad. Sci. USA* **73**, 2584–2588
8. Brown-Luedi, M.L., Meyer, L. J., Milburn, S.C., Yau, P.M., Corbett, S., and Hershey, J.W. (1982) Protein synthesis initiation factors from human HeLa cells and rabbit reticulocytes are similar: comparison of protein structure, activities, and immunochemical properties. *Biochemistry* **21**, 4202–4206
9. Hershey, J.W., Asano, K., Naranda, T., Vornlocher, H.P., Hanachi, P., and Merrick, W.C. (1996) Conservation and diversity in the structure of translation initiation factor EIF3 from humans and yeast. *Biochimie* **78**, 903–907
10. Danaie, P., Wittmer, B., Altmann, M., and Trachsel, H. (1995) Isolation of a protein complex containing translation initiation factor Prt1 from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **270**, 4288–4292
11. Naranda, T., MacMillan, S.E., and Hershey, J.W. (1994) Purified yeast translational initiation factor eIF-3 is an RNA-binding protein complex that contains the PRT1 protein. *J. Biol. Chem.* **269**, 32286–32292
12. Methot, N., Rom, E., Olsen, H., and Sonenberg, N. (1997) The human homologue of the yeast Prt1 protein is an integral part of the eukaryotic initiation factor 3 complex and interacts with p170. *J. Biol. Chem.* **272**, 1110–1116
13. Feinberg, B., McLaughlin, C.S., and Moldave, K. (1982) Analysis of temperature-sensitive mutant ts 187 of *Saccharomyces cerevisiae* altered in a component required for the initiation of protein synthesis. *J. Biol. Chem.* **257**, 10846–10851
14. Hanic-Joyce, P.J., Johnston, G.C., and Singer, R.A. (1987) Regulated arrest of cell proliferation mediated by yeast prt1 mutations. *Exp. Cell Res.* **172**, 134–145
15. Gossler, A., Joyner, A.L., Rossant, J., and Skarnes, W.C. (1989) Mouse embryonic stem cells and reporter constructs to detect developmentally regulated genes. *Science* **244**, 463–465
16. Baker, R.K., Haendel, M.A., Swanson, B.J., Shambaugh, J.C., Micales, B.K., and Lyons, G.E. (1997) *In vitro* preselection of gene-trapped embryonic stem cell clones for characterizing novel developmentally regulated genes in the mouse. *Dev. Biol.* **185**, 201–214
17. Kondoh, G., Yamamoto, Y., Yoshida, K., Suzuki, Y., Osuka, S.,

Nakano, Y., Morita, T., and Takeda, J. (1999) Easy assessment of ES cell clone potency for chimeric development and germline competency by an optimized aggregation method. *J. Biochem. Biophys. Methods* **39**, 137–142

18. Miyashita, A., Shimizu, N., Endo, N., Hanyuu, T., Ishii, N., Ito, K., Itoh, Y., Shirai, M., and Nakajima, T. (1999) Five different genes, Eif4a1, Cd68, Supl15h, Sox15 and Fxr2h, are clustered in a 40 kb region of mouse chromosome 11, *Gene* **237**, 53–60