

antigen-specific deletion of CD4<sup>+</sup>8<sup>+</sup> thymocytes indicates that it is not a unique property of the thymic microenvironment, but is the property of a certain developmental stage of T cells which makes it sensitive to deletion. This agrees with the recent study in thymic organ cultures from normal mice, in which the ability of peripheral dendritic cells to induce unresponsiveness in developing T cells by an unknown mechanism was shown<sup>15</sup>. Thus, antigens carried on APCs from the periphery into the thymus may induce central tolerance. The demonstration of antigen-specific deletion of CD4<sup>+</sup>8<sup>+</sup> thymocytes in suspension culture offers new possibilities in the study of the selection mechanisms operating at this critical stage of T-cell development<sup>16</sup>. In particular, the role of various T-cell receptors and their ligands (for example, TCR, CD4, CD8, antigenic peptides<sup>17</sup> and MHC molecules), as well as the role of different stromal cells, in determining whether deletion (apoptosis) or positive selection (inhibition of apoptosis) will result from interaction of immature thymocytes with thymic microenvironment, may be directly approached in a defined *in vitro* system. □

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- Kappler, J. W., Roehm, N. & Marrack, P. C. *Cell* **49**, 273-280 (1987).
- MacDonald, H. R. *et al. Nature* **332**, 40-45 (1988).
- Kisielow, P., Blüthmann, H., Staerz, U., Steinmetz, M. & von Boehmer, H. *Nature* **333**, 742-746 (1988).
- Sha, W. C. *et al. Nature* **335**, 271-274 (1988).
- Fowlkes, B. J., Schwartz, R. H. & Pardoll, D. M. *Nature* **334**, 620-623 (1988).
- MacDonald, H. R., Hengartner, H. & Pedrazzini, M. *Nature* **335**, 174-176 (1988).
- Jenkins, E. J. *et al. Eur. J. Immunol.* **19**, 2175-2177 (1989).
- von Boehmer, H. *et al. Rev. Immunol.* **8**, 531-556 (1990).
- MacDonald, H. R. & Lees, R. K. *Nature* **343**, 642-644 (1990).
- Longo, D. L. & Schwartz, R. H. *Nature* **287**, 44-46 (1980).
- Swat, W., Ignatowicz, L. & Kisielow, P. *J. Immunol. Meth.* **137**, 79-97 (1991).
- Teh, H. S. *et al. Devl Immunol.* **1**, 1-10 (1990).
- Ceredig, R., Dialynas, D. P., Fitch, F. W. & MacDonald, H. R. *J. exp. Med.* **158**, 1654-1660 (1983).
- White, J. *et al. Cell* **56**, 27-34 (1989).
- Matzinger, P. & Guelder, S. *Nature* **338**, 74-76 (1989).
- von Boehmer, H. & Kisielow, P. *Science* **248**, 1369-1373 (1990).
- Rotzschke, O. *et al. Science* **249**, 283-287 (1990).
- Smith, C. A. *et al. Nature* **337**, 181-183 (1989).

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## Parental imprinting of the mouse H19 gene

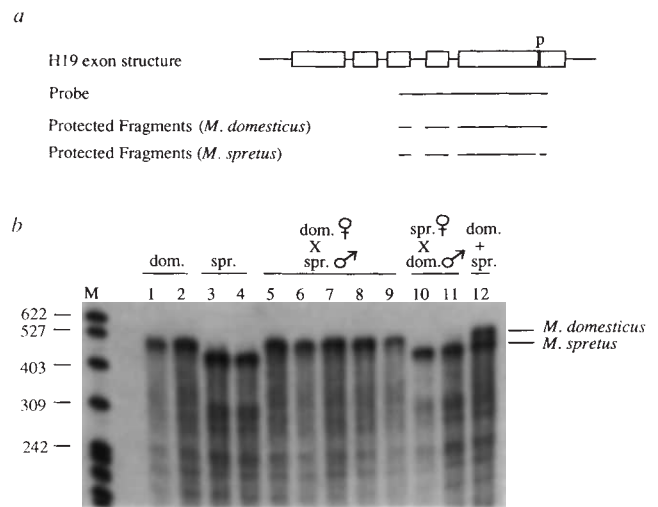
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**THE mouse *H19* gene encodes one of the most abundant RNAs in the developing mouse embryo<sup>1</sup>. It is expressed at the blastocyst stage of development, and accumulates to high levels in tissues of endodermal and mesodermal origin (H. Kim, unpublished result). After birth the gene is repressed in all tissues except skeletal muscle. It lacks a common open reading frame in the 2.5-kilobase RNA, but has considerable nucleotide sequence similarity between the genes of rodents and humans<sup>2,3</sup>. Expression of the gene in transgenic mice results in late prenatal lethality, suggesting that the dosage of its gene product is strictly controlled<sup>4</sup>. The *H19* gene maps to the distal segment of mouse chromosome 7, in a region that is parentally imprinted<sup>5</sup>, a process by which genes are differentially expressed on the maternal and paternal chromosomes. We have now used an RNase protection assay that can distinguish between *H19* alleles in four subspecies of *Mus*, to demonstrate that the *H19* gene is parentally imprinted, with the active copy derived from the mother. This assay will be of general use in assaying allele-specific gene expression.**

To determine whether a gene is imprinted, one must be able

to distinguish expression from the maternal and paternal alleles. We used an RNase protection assay that is very sensitive to base substitutions and discontinuities in RNA sequences<sup>6</sup>. Hybrid F<sub>1</sub> mice generated from an interspecies cross between *Mus musculus domesticus* and *Mus spretus* were assayed for RNA levels transcribed from each *H19* allele. These two species diverged in evolution between 3 to 6 million years ago<sup>7</sup>, and exhibit significant nucleotide polymorphisms, a property that has been especially useful in genetic mapping strategies<sup>8</sup>. For the *H19* gene, the fifth exon is especially divergent in sequence between humans and mice<sup>3</sup>, and provides a likely candidate for harbouring an RNA polymorphism. An antisense RNA probe that included the 3' end of exon 3, all of exon 4 and the 5' part of exon 5 was derived from genomic DNA of the *M. domesticus* strain BALB/cJ (Fig. 1a). After hybridization to total liver RNA isolated from *M. domesticus* and *M. spretus* neonates, the RNase-resistant products were analysed by gel electrophoresis. *M. domesticus* RNA protected the expected full-length fragments corresponding to exons 3, 4 and 5. *M. spretus* RNA also fully protected exons 3 and 4 (date not shown), but a slightly



**FIG. 1** RNase protection assay of liver RNA from *M. domesticus*, *M. spretus* and hybrid animals. *a*, The top line shows the exon structure of the mouse *H19* gene, drawn in a 5' to 3' orientation. Open boxes indicate exons and the lines correspond to introns and nontranscribed regions. The filled box in exon 5 and the corresponding 'p' above the box marks one possible site of the RNA polymorphism that exists between *M. domesticus* (dom.) and *M. spretus* (spr.). The second line shows a *Bam*HI-*Stu*I 754-base pair (bp) genomic DNA fragment that was used as the RNase protection probe. The probe fragments that are protected from RNase digestion following hybridization with *M. domesticus* or *M. spretus* H19 RNA are drawn in the third and fourth lines, respectively. *b*, Autoradiogram of RNase protection assay products of 2 μg total liver RNA samples from neonatal mice. The F<sub>1</sub> progeny of *M. domesticus* females mated to *M. spretus* males (lanes 5-9) and of *M. spretus* females mated to *M. domesticus* males (lanes 10 and 11), as well as the parental lines *M. domesticus* (lanes 1 and 2) and *M. spretus* (lanes 3 and 4), are as indicated. Only the allele-specific protected fragment from exon 5 is included. Marker lane (M) contains radioactively labelled DNA fragments of *Msp*I-digested pBR322, with relative sizes indicated in base pairs. Lane 12 shows an assay control using 1 μg *M. domesticus* RNA and 1 μg *M. spretus* RNA. As both protected fragments display comparable intensities, the probe is equally capable of detecting both types of RNA. **METHODS.** The RNase protection was performed as described<sup>6</sup> with the following exceptions. The *Bam*HI-*Stu*I fragment of the *H19* gene was cloned into the *Bam*HI-*Hinc*II sites of pBluescript II KS (Stratagene). The vector was linearized with *Xba*I, purified by agarose gel electrophoresis and used as a template in an *in vitro* transcription reaction using [<sup>32</sup>P]CTP and T3 polymerase (Promega). Total RNA was prepared from day 2-5 neonatal animals by the lithium chloride/urea method<sup>19</sup> and hybridized to 1 × 10<sup>5</sup> c.p.m. of radiolabelled probe overnight at 65°C. After RNase digestion at 32°C for 1 h, samples were analysed by electrophoresis on a 6.6% acrylamide, 7 M urea gel, and exposed to Kodak XAR-5 X-ray film.

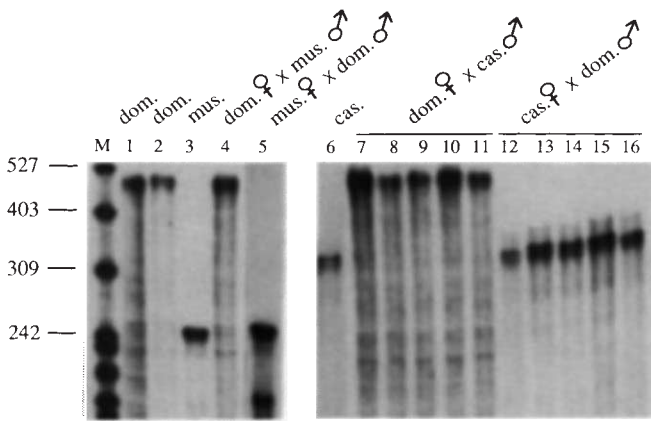


FIG. 2 RNase protection assay of skeletal muscle RNA from the progeny of *M. domesticus* crossed to either *M. castaneus* (cas.) or *M. musculus* (mus.). Total RNAs were isolated from skeletal muscle of 4-day-old *M. domesticus* (BTBR strain, 2 µg; lane 1), 3-month-old *M. domesticus* (CBA/NJX10/X10 strain, 10 µg; lane 2), 6-week-old *M. musculus* (SKIVE strain, 10 µg; lane 3), a 4-month-old F<sub>1</sub> mouse derived from a *M. domesticus* (CBA/NJX10/X10) female and *M. musculus* (Denmark) male cross (10 µg, lane 4) and a 6-week-old F<sub>1</sub> mouse from the reciprocal cross (10 µg, lane 5). Each RNA sample was subjected to the RNase protection assay, as described in the legend to Fig. 1. Total RNA derived from a *M. castaneus* neonate (1 µg, lane 6), individual 3-week-old progeny of *M. domesticus* (C57BL/6J strain) females crossed to *M. castaneus* males (5 µg, lanes 7–11) and progeny of *M. castaneus* females mated to *M. domesticus* males (lanes 12–16) were treated similarly. The protected fragments correspond to exon 5.

smaller exon 5 fragment was observed (Fig. 1b, compare lanes 1 and 2 to lanes 3 and 4).

When liver RNAs from neonatal progeny of *M. domesticus* females mated with *M. spretus* males were tested with the RNase protection assay, only the *H19* gene product characteristic of *M. domesticus* exon 5 was observed (Fig. 1b, lanes 5–9). Conversely, the reciprocal cross using *M. domesticus* males and *M. spretus* females resulted in progeny that expressed only the *M. spretus* *H19* allele (Fig. 1b, lanes 10 and 11). In both cases, only the allele inherited from the mother was expressed.

The exclusive expression of the *H19* maternal allele could be

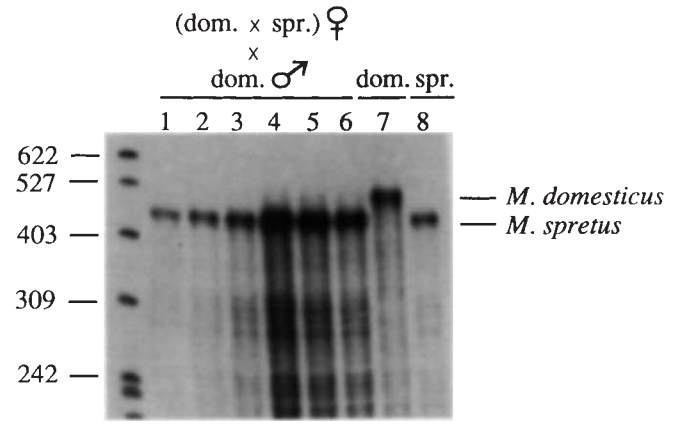


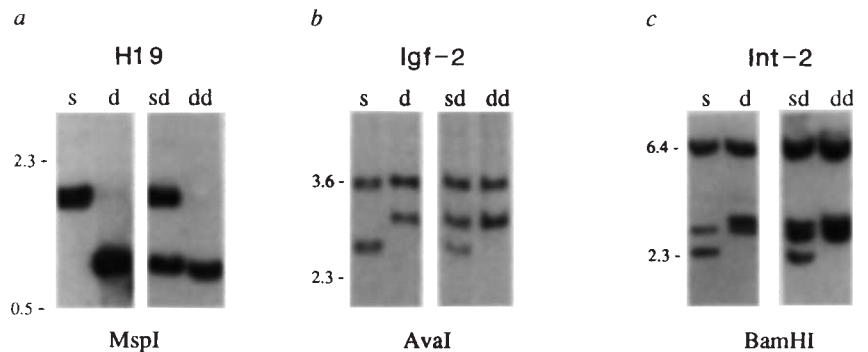
FIG. 3 Analysis of liver RNAs from *M. domesticus* × *M. spretus* backcross progeny. Female F<sub>1</sub> mice from a *M. domesticus* female × *M. spretus* male cross (Fig. 1) were mated with *M. domesticus* males and progeny were killed at different ages after birth. Total liver RNA isolated from progeny identified as being heterozygous for *H19* were assayed by RNase protection as described in Fig. 1: 1 day (1 µg, lanes 1 and 2), 4 days (2 µg, lane 3), and 1 week (5 µg, lanes 4–6). Liver RNA from homozygous *M. domesticus* and *M. spretus* was also assayed (2 µg, each, lanes 7 and 8, respectively). The protected fragments correspond to exon 5.

a unique property of the mouse strains used in this interspecific cross. We therefore analysed two additional crosses using different *Mus* species. Skeletal muscle RNAs from 2–3-month-old progeny from *M. domesticus* × *Mus musculus musculus* reciprocal crosses were assayed for the presence of *H19* RNA. The *M. musculus* RNA protects a fragment of about 240 bases, easily distinguished from the *M. domesticus* product (Fig. 2, lanes 1–3). Analysis of the hybrid progeny confirmed that only the maternally derived allele was expressed (Fig. 2, lanes 4 and 5).

Likewise, when skeletal muscle RNAs derived from F<sub>1</sub> progeny of *M. domesticus* × *Mus musculus castaneus* reciprocal crosses were analysed, the maternal allele was once again exclusively detected (Fig. 2, lanes 7–16). In addition to verifying the parental imprinting of the *H19* gene, these experiments illustrate that cells of both liver and skeletal muscle, derived from two

FIG. 4 Genetic mapping of the *H19* gene. a, b and c, Southern blots identifying RFLPs between *M. spretus* and *M. domesticus* using a 1.1-kb *Bam*H1 *H19* genomic DNA fragment<sup>3</sup>, a 550-bp rat *Igf-2* cDNA<sup>20</sup>, and a 1.0-kb mouse *Int-2* cDNA<sup>21</sup>, respectively. In each panel the first two lanes show *M. spretus* (s) and *M. domesticus* (d; BALB/cJ strain) parental DNAs and the last two lanes show heterozygous (sd) and homozygous (dd) DNA from a (s × d) × d backcross. The restriction endonuclease used to digest the DNA is designated under each panel and relative size standards (in kb) are shown to the left. d, Number of recombinants detected between the *Int-2* and *H19* or *Igf-2* genes. The genetic distance represents the maximum distance defined at the 95% confidence level, calculated using the formula  $0.05 = (1 - p)^N$ , where *p* is map distance in Morgans and *N* is number of animals tested.

**METHODS.** Genomic DNA (15 µg) was digested and separated on a 1.0% agarose gel. DNA was transferred to Genescreen (NEN) by capillary transfer in 0.2 N NaOH/0.6 N NaCl. The filters were neutralized in 1 M Tris-HCl (pH 7.5), hybridized overnight using the conditions described by Church and Gilbert<sup>22</sup> and washed for 2 h at 65°C in 0.015 M NaCl-0.0015 M sodium citrate/0.1% SDS. The probes were prepared by the method of Feinberg and Vogelstein<sup>23</sup> using [<sup>32</sup>P]dCTP.



	Recombination interval	Number of recombinants	Map distance (cM)
d	<i>H19</i> - <i>Int-2</i>	0/110	<2.7
	<i>Igf-2</i> - <i>Int-2</i>	0/109	<2.7



different germ layers, display similar imprinting patterns, as does the neonatal gut (data not shown).

If the *H19* gene is indeed parentally imprinted, the pattern of allele-specific expression will be reset in the germ line at each generation. To test this, female  $F_1$  (*M. domesticus* × *M. spretus*) mice, expressing the *M. domesticus* *H19* allele (Fig. 1b, lanes 5–9), were backcrossed to *M. domesticus* males. The progeny that inherited the *M. spretus* *H19* allele from the mother were identified by restriction fragment length polymorphism analysis of genomic DNA (data not shown). Analysis by RNase protection of liver RNAs from the progeny demonstrated that the maternally derived *M. spretus* allele was exclusively expressed (Fig. 3, lanes 1–6). This result demonstrates that the formerly silent *M. spretus* allele inherited from the mother was reactivated during oogenesis. A total of 24 backcross progeny were screened, and all displayed the exclusive expression of the *M. spretus* maternal allele. From this we also conclude that the imprinting requires sequences that are linked to the *H19* gene itself. In addition, the segregation of unlinked modifiers of imprinting, which can occur for imprinted transgenes<sup>9,10</sup>, was not detected.

Both the *H19* gene<sup>1</sup> and the insulin-like growth factor-II gene (*Igf-2*) (T. Glaser and D. Housman, personal communication) map to mouse chromosome 7. Recently DeChiara *et al.* deleted the *Igf-2* gene by homologous recombination in embryonic stem cells<sup>11</sup>. Heterozygotes who had inherited this inactive *Igf-2* gene from the father were smaller than normal. When the inactive gene was inherited from the mother, however, the heterozygote progeny were of normal size<sup>12</sup>. The authors conclude that only the paternal allele of *Igf-2* is active in most expressing tissues, with the exception of the choroid plexus and the leptomeninges, where both alleles are active.

We used an interspecific backcross between the *M. domesticus* BALB/cJ strain and *M. spretus* to determine the genetic linkage between *H19* and *Igf-2*. Restriction fragment length polymorphisms that distinguish the alleles of the two genes (Fig. 4a–c) were assayed in the genomic DNAs of 110 progeny of a (BALB/cJ × *M. spretus*) × BALB/cJ backcross. No recombinants were observed (Fig. 4d). To position the genes on the chromosome 7 map, they were mapped relative to *Int-2*, which encodes a member of the fibroblast growth factor gene family, and which has been mapped to the distal third of chromosome 7 (ref. 13). No recombinants between *Int-2*, *H19* and *Igf-2* were detected, indicating that these genes are tightly linked. *H19* and *Igf-2* have been mapped to band 11p15.5 in the human genome<sup>14</sup>. This syntenic conservation would tend to discount our failure to detect recombinants being the consequence of an inversion on distal chromosome 7 of *M. spretus* relative to *M. domesticus*<sup>15</sup>.

That the *H19* and *Igf-2* genes are tightly linked, yet imprinted in opposite directions, implies that the signals for parental imprinting are unlikely to act over large chromosomal regions, in contrast to X chromosome inactivation<sup>16</sup>. Indeed, Barlow *et al.* showed that the *Igf-2r* gene, encoding the receptor for insulin-like growth factor-II, is expressed only from the maternally inherited chromosome 17 (ref. 17). But three other genes within a region of about 1.1 megabases containing *Igf-2r* are not subject to imprinting.

That the *H19* gene undergoes parental imprinting was suggested by two lines of evidence. First, introduction of additional copies of the *H19* gene in transgenic mice leads to late prenatal lethality, between 14 and 16 days of gestation<sup>4</sup>. Second, *H19* maps to a region which is parentally imprinted<sup>5</sup>. Using balanced translocations, Searle and Beechey demonstrated that a maternal duplication/paternal deficiency of distal chromosome 7 results in late prenatal lethality, whereas paternal duplication/maternal deficiency leads to early embryonic lethality<sup>18</sup>. The remarkable similarity in the timing of the lethal effects of extra copies of the *H19* gene in the transgenic experiments and the maternal duplications of chromosome 7 suggest strongly that they share the same aetiology: one or more extra copies of an active *H19*

gene. Whether this is the true cause of the lethality remains to be determined. The absence of *Igf-2* gene function, which would be the case in mice carrying the maternal duplications/paternal deficiencies, results in smaller mice, and therefore it alone cannot explain the prenatal lethality<sup>12</sup>.

Whether the early embryonic lethality observed with paternal duplications/maternal deficiencies can be explained by loss of *H19* gene function will require the generation of such mutations through gene replacement in embryonic stem cells. Nevertheless, the studies described here are consistent with an important role for the *H19* gene in embryonic development in the mouse. That role remains unclear, however, given the unusual nature of the gene product, which is found in a highly abundant cytoplasmic RNA complex<sup>3</sup>. That even one extra copy of the gene may have specific deleterious effects implies that its abundance is being carefully controlled. □

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- Pachnis, V., Belayew, A. & Tilghman, S. M. *Proc. natn. Acad. Sci. U.S.A.* **81**, 5523–5527 (1984).
- Pachnis, V., Brannan, C. I. & Tilghman, S. M. *EMBO J.* **7**, 673–681 (1988).
- Brannan, C. I., Dose, F. C., Ingersoll, R. S. & Tilghman, S. M. *Mol. Cell Biol.* **10**, 28–36 (1990).
- Brunkow, M. E. & Tilghman, S. M. *Genes Dev.* (in the press).
- Searle, A. G. & Beechey, C. V. in *Aneuploidy* (eds Dellarcio, V. L., Voytek, P. E. & Hollaender, A.) 363–376 (Plenum, New York/London, 1985).
- Melton, D. A. *et al. Nucleic Acids Res.* **12**, 7035–7056 (1984).
- Bonhomme, F. *et al. Biochem. Genet.* **22**, 275–303 (1984).
- Robert, B. *et al. Nature* **314**, 181–183 (1985).
- Sapienza, C., Paquette, J., Tran, T. H. & Peterson, A. *Development* **107**, 165–168 (1989).
- Allen, N. D., Norris, M. L. & Surani, M. A. *Cell* **61**, 853–861 (1990).
- DeChiara, T. M., Efstratiadis, A. & Robertson, E. J. *Nature* **345**, 78–80 (1990).
- DeChiara, T. M., Robertson, E. J. & Efstratiadis, A. *Cell* **64**, 849–859 (1991).
- Davison, M. T., Roderick, T. H., Hillyard, A. L. & Doolittle, D. P. *Mouse Newslett.* **81**, 12–19 (1988).
- Glaser, T., Housman, D., Lewis, W. H., Gerhard, D. & Jones, C. *Somatic cell. molec. Genet.* **15**, 477–501 (1989).
- Hammer, M. F., Schimenti, J. & Silver, L. M. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3261–3265 (1989).
- Lyon, M. F. *Am. J. hum. Genet.* **42**, 8–16 (1988).
- Barlow, D. P., Stoger, R., Herrmann, B. G., Saito, K. & Schweifer, N. *Nature* **349**, 84–87 (1991).
- Searle, A. G. & Beechey, C. V. *Genet. Res.* **56**, 237–244 (1990).
- Auffray, C. & Rougeon, F. *Eur. J. Biochem.* **107**, 303–314 (1980).
- Soares, M. B. *et al. J. molec. Biol.* **192**, 737–752 (1986).
- Wilkinson, D. G., Peters, G., Dickson, C. & McMahon, A. P. *EMBO J.* **7**, 691–695 (1988).
- Church, G. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1991–1995 (1984).
- Feinberg, A. P. & Vogelstein, B. *Analyt. Biochem.* **132**, 6–13 (1983).

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## Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor

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HUMAN exposure to *Pneumocystis carinii* is common<sup>1,2</sup> but, in the absence of acquired<sup>3</sup> or genetic<sup>4</sup> dysfunction of either cellular or humoral immunity, exposure rarely leads to illness. Although alveolar macrophages can degrade *P. carinii*<sup>5,6</sup>, macrophage receptors involved in *P. carinii* recognition have not been clearly defined.