signal that induces the migration of myogenic precursor cells and could possibly play an additional role as a chemo-attractant for migrating cells, that is, direct them to the target.

The c-met receptor tyrosine kinase was first identified because of its oncogenic potential when mutated[20]. The specific ligand that binds to c-met and regulates the tyrosine kinase activity of the receptor is SF/HGF. The first activities described for this factor were the induction of motility of epithelial Madin Darby canine kidney cells[5][7] (scatter factor) and induction of growth of primary hepatocytes[4] (hepatocyte growth factor). In vitro, SF/HGF also induces invasion and migration in extracellular matrix[21], a process reminiscent of cell migration in vivo. Other responses to SF/HGF include tubular morphogenesis[22] and not only epithelial, but also endothelial and haematopoietic cells have been reported to respond to the factor[23,24]. Our genetic studies demonstrate a complex role for c-met in the control of growth, survival and migration of distinct embryonal cells. Processes that depend on SF/HGF and c-met require signal exchange between different cellular compartments that are located in close vicinity. The high affinity of SF/HGF for heparin, which is present on cell surfaces and extracellular matrix, might limit the diffusion and thus allow local actions of the factor only. The indistinguishable phenotypes we observe in mice with targeted mutations in SF/HGF or c-met also demonstrate that maternal SF/HGF can not diffuse and compensate for locally produced, embryonal factor, not even in the placenta. □

1. Christ, B., Jacob, H. & Jacob, M. Anat. Embryol. **150**, 171–186 (1977).
2. Chevallier, A., Kieny, M. & Mauger, A. J. Embryol. exp. Morph. **41**, 245–258 (1977).
3. Hayashi, K. & Ozawa, E. Development **121**, 661–669 (1995).
4. Nakamura, T. et al. Nature **342**, 440–443 (1989).
5. Stoker, M., Gherardi, E., Perryman, M. & Gray, J. Nature **327**, 239–242 (1987).
6. Gherardi, E., Gray, J., Stoker, M., Perryman, M. & Furlong, R. Proc. natn. Acad. Sci. U.S.A. **86**, 5844–5848 (1989).
7. Weidner, M. et al. Proc. natn. Acad. Sci. U.S.A. **88**, 7001–7005 (1991).
8. Bottaro, D. et al. Science **251**, 802–804 (1991).
9. Naldini, L. et al. Oncogene **6**, 501–504 (1991).
10. Birchmeier, C. & Birchmeier, W. A. Rev. Cell Biol. **9**, 511–540 (1993).
11. Schmidt, C. et al. Nature **373**, 699–702 (1995).
12. Uehara, Y. et al. Nature **373**, 702–705 (1995).
13. Papaioannou, V. & Johnson, R. in Gene Targeting: A Practical Approach (ed. Joyner, A.) 107–146 (IRL, Oxford, 1994).
14. Lyons, G. & Buckingham, M. Semin. dev. Biol. **3**, 243–253 (1992).
15. Sassoon, D. et al. Nature **341**, 303–307 (1989).
16. Ordahl, C. & Le Douarin, N. Development **114**, 339–353 (1992).
17. Bober, E., Franz, T., Arnold, H., Gruss, P. & Temblay, P. Development **120**, 603–612 (1994).
18. Goulding, M., Lumsden, A. & Paquette, A. Development **120**, 957–971 (1994).
19. Williams, B. & Ordahl, C. Development **120**, 785–796 (1994).
20. Park, M. et al. Cell **25**, 895–904 (1986).
21. Weidner, M., Sachs, M. & Birchmeier, W. J. Cell Biol. **121**, 145–154 (1993).
22. Montesano, R., Matsumoto, K., Nakamura, T. & Orci, L. Cell **67**, 901–908 (1991).
23. Rosen, E., Nigam, S. & Goldberg, I. J. Cell Biol. **127**, 1783–1787 (1994).
24. Zarnegar, R. & Michalopoulos, G. J. Cell Biol. **129**, 1177–1180 (1995).
25. Mansour, S., Thomas, K. & Capecchi, M. Nature **336**, 348–352 (1988).
26. Gonzatti-Haces, M. et al. Proc. natn. Acad. Sci. U.S.A. **85**, 21–25 (1988).
27. Yenofsky, R., Fine, M. & Pellow, J. Proc. natn. Acad. Sci. U.S.A. **87**, 3435–3439 (1990).
28. Kuehn, R., Rajewski, K. & Mueller, W. Science **254**, 707–710 (1991).
29. Iyer, A. et al. Cell Growth Differ. **1**, 87–95 (1990).
30. Sonnenberg, E., Meyer, D., Weidner, M. & Birchmeier, C. J. Cell Biol. **123**, 223–235 (1993).

Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C

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GROWTH is one of the fundamental aspects in the development of an organism. Classical genetic studies have isolated four viable, spontaneous mouse mutants[1] disrupted in growth, leading to dwarfism. Pygmy is unique among these mutants because its phenotype cannot be explained by aberrations in the growth hormone–insulin-like growth factor endocrine pathway[2-5]. Here we show that the pygmy phenotype arises from the inactivation of Hmgi-c (ref. 6), a member of the Hmgi family[7] which function as architectural factors in the nuclear scaffold[8] and are critical in the assembly of stereospecific transcriptional complexes[9]. Hmgi-c and another Hmgi family member, Hmgi(y) (ref. 10), were found to be expressed predominantly during embryogenesis. The HMGI proteins are known to be regulated by cell cycle-dependent phosphorylation which alters their DNA binding affinity[11]. These results demonstrate the important role of HMGI proteins in mammalian growth and development.

The first step in the molecular definition of the pygmy mutation was made possible by the isolation of a transgenic insertional mouse mutant at the locus, $pg^{TgN40AChu}$ (ref. 12). A 0.5-kb ApaI-ApaI single-copy genomic sequence 2 kb from the site of transgene insertion was identified[12] and used to initiate a bi-directional chromosome walk on normal mouse genomic DNA. The analysis of seven overlapping clones spanning 91 kb delineated a 56-kb common deletion between two informative mutants, pg and $pg^{TgN40AChu}$ (Fig. 1a).

The common area of disruption was investigated further for candidate transcription units. The technique of exon amplification[13] was used to identify putative exons, and clones 803 and 5B, in the same orientation, produced spliced products (Fig. 1b). Their sequence was determined[14], and a comparison with DNA sequence databases (GenBank and EMBL) revealed 100% homology to a previously identified gene, Hmgi-c (ref. 6) (Fig. 1c). Hmgi-c belongs to the Hmgi family of the general class of HMG (high mobility group) DNA-binding proteins[7]. The HMGI proteins have been assigned multiple functions[6] and have been shown to be important in the regulation of gene expression as architectural factors by inducing DNA conformational changes in the formation of the three-dimensional transcription complex[15,16].

The genomic structure of Hmgi-c revealed that it contains five exons and spans a region of approximately 110 kb (Fig. 1d). Single-copy sequences from the 190-kb cloned pygmy locus, surrounding and including the Hmgi-c gene (Fig. 1d), were used as probes on Southern blots containing DNA isolated from the two informative alleles[12]. The genomic area encompassing Hmgi-c is completely deleted in the transgenic insertional mutant $pg^{TgN40AChu}$ (A/A), whereas in the spontaneous mutant pg the 5' sequences and the first two exons are absent (Fig. 1d).

Previous studies[17] had established that the pygmy phenotype could be observed at birth. Therefore, RNA from wild-type mouse embryos was isolated[18], and northern blot analysis revealed a transcript of 4.1 kb (Fig. 2). As expected from the genomic analysis, no detectable Hmgi-c expression was observed in the spontaneous and transgenic insertional mouse mutants. A third allele also exists at the pygmy locus[1], In(10)17Rk,

which carries an inversion of chromosome 10, and the distal breakpoint is within intron 3 of the *Hmgi-c* gene (data not shown). No *Hmgi-c* expression was detected in homozygous embryonic *In(10)17Rk* RNA (Fig. 2). Quantification by phosphorimager analysis showed that heterozygous mice expressed *Hmgi-c* at approximately 50% of wild-type levels. Therefore the wild-type allele in the heterozygous mice does not increase its expression levels to compensate for the loss of the deleted allele. This is consistent with the pygmy mutation being semi-dominant because there is a mild phenotypic effect on heterozygous mice (80% of the weight of wild-type mice)[19]. Furthermore, *Hmgi(y)*, the only other known member of the *Hmgi* gene family[7], retained the same levels of expression in the mutant and wild-type mice (Fig. 2). Thus there is no
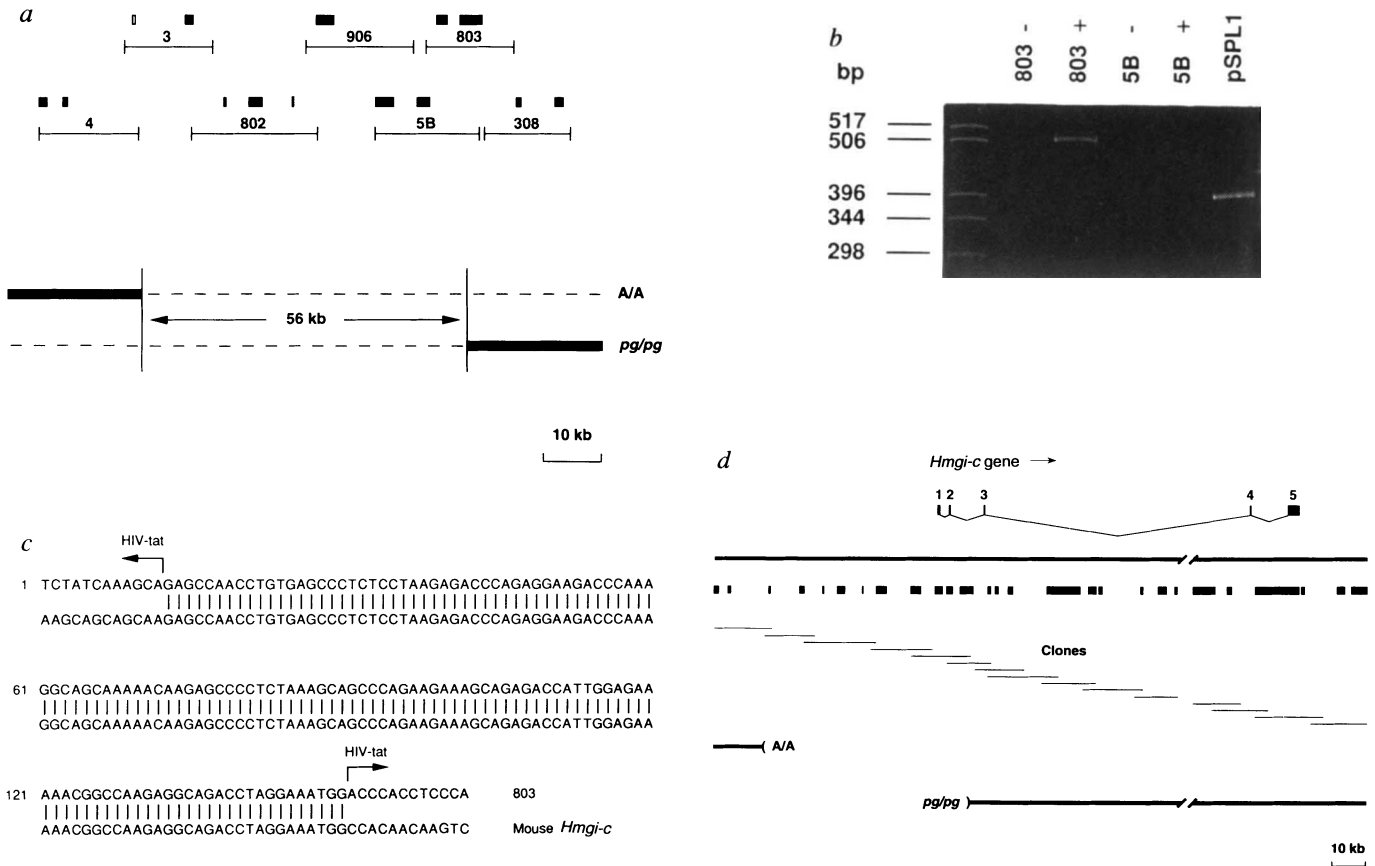

FIG. 1 Identification and genomic characterization of the *Hmgi-c* gene at the pygmy locus in normal and mutant alleles. *a*, Delineation of the overlapping deleted genomic regions at the pygmy locus in the spontaneous and transgenic insertional mouse mutants. The open box above clone 3 positions the 0.5-kb *ApaI-ApaI* fragment, and the filled boxes represent single copy sequences used as probes to analyse genomic DNA isolated from mice of varying genotypes[12]. Solid and broken lines represent presence or absence of genomic sequences, respectively, in the transgenic insertional mouse mutant $pg^{TgN4OACha}$ (A) and the spontaneous mutant pygmy (pg). *b*, Exon amplification from lambda clones 803 and 5B. The primary polymerase chain reaction (PCR) exon amplification products in both sense (+) and antisense (−) orientations from the lambda clones shown in *a* were analysed on a 5% polyacrylamide gel[13]. The 379-bp PCR product observed in the control pSPL1 lane results from splicing between the HIV *tat* and β-globin vector sequences[13]. *c*, Sequence of exons amplified from clone 803 and comparison with the *Hmgi-c* gene. *d*, A series of overlapping phage clones extending approximately 190 kb at the pygmy locus. The discontinuous region represents an unclonable 11-kb fragment, as estimated from Southern blots of cleaved genomic DNA probed with single copy sequences from the end of the clonable region. The position and number of the *Hmgi-c* exons (not drawn to scale) are shown above the wild-type locus. Single copy sequences were isolated at the indicated positions and are represented by filled boxes below the wild-type locus. Thick bars and blank regions represent the genomic sequences that are present or deleted in the two alleles.

METHODS. The 0.5-kb *ApaI-ApaI* fragment[12] was used as a probe to isolate clones 3 and 4 from an EMBL3 mouse genomic library (a gift from E. Lacy) and a YAC (902C0711) from a mouse YAC library[22]. YAC 902C0711 was further subcloned into lambda FIX II (ref. 14), and 86

clones that hybridized to radioactively labelled mouse genomic DNA were picked and transferred to new plates in a grid array[14]. Lambda clones 802, 906, 5B, 803 and 308 were isolated after the walk was initiated with the 0.5-kb *ApaI-ApaI* fragment and accomplished by repeated hybridization to filters of the array. Overlaps between the contiguity clones and collinearity with the genome were confirmed by a combination of clone to clone and clone to genomic hybridizations and with restriction mapping. Exon amplification was done (Exon Trapping System, Gibco BRL) after the genomic inserts from the lambda clones were removed by cleavage with *SalI*, partly filled in[14] and subcloned into a partly filled-in *Bam*H1 cleaved pSPL1 plasmid[13]. The DNA was electroporated into COS-7 cells at 180 V and 960 μF in a Bio-Rad Gene Pulser. Cytoplasmic RNA was isolated after 2-3 days and RT–PCR performed using primers supplied by the manufacturer. The secondary PCR amplification products[13] from clones 803 and 5B were subcloned into the plasmid vector, pAMP10 (Exon Trapping System, Gibco BRL) and sequenced using the Sequenase Version 2.0 sequencing kit (USB)[14]. A 344-bp fragment corresponding to the complete open reading frame of the *Hmgi-c* gene[6] was amplified from 12.5 d.p.c. mouse embryos (see text) using RT–PCR. Lambda clones containing the *Hmgi-c* exons were then isolated by hybridization of the 344-bp radioactively labelled fragment to the grid array of lambda clones and subsequently connected through chromosome walking. The RT– PCR conditions for isolation of the 344-bp fragment consisted of first strand cDNA synthesis with primer 1 (5′-ATGAATTCCTAATCCTCCTCTGC-3′), followed by PCR amplification with primers 1 and 2 (5′-ATGGATCCATGACGCACGCGGT-3′). PCR conditions were 94 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 1 min, for 30 cycles. The amplified product was confirmed by sequencing analysis[14].

FIG. 2 Hmgi-c gene expression of three alleles at the mouse pygmy locus. The wild-type allele is represented by +, the transgenic allele $pg^{TgN4OACha}$ by A, the spontaneous mutant allele by pg, and an allele at the pygmy locus which involves a paracentric inversion on chromosome 10 (In(10)17Rk) by Rk.

METHODS. The genotypes were established for mice in line A and the spontaneous mutant pg as previously described[12], and mice containing the In(10)17Rk inversion were detected by a PCR-based restriction fragment length polymorphism (L. Cherath, K.F.B. and K.C., in preparation). RNA was isolated from 12.5 d.p.c. embryos, and equal amounts (5 μg) were analysed by northern blot hybridization[14]. The probes were a 138-bp nucleotide cDNA fragment encompassing exons 2 and 3 of the Hmgi-c gene, and a 340-bp cDNA fragment containing the complete coding sequence of the Hmgi(y) gene[10]. The blot was subsequently hybridized to a oligonucleotide complementary to murine 28S ribosomal RNA[23] to ensure that equal amounts of RNA were present in each lane, and the results are shown in the lower panel.
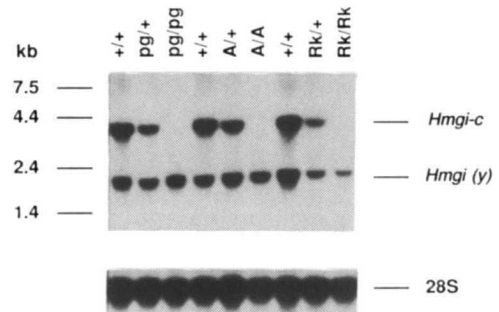
compensation by Hmgi(y) for the lack of Hmgi-c expression in pygmy mice.

The mutant alleles described above arise from major disruptions of genomic DNA which result in large deletions or a chromosomal inversion. To exclude the possibility that a gene other than Hmgi-c may be responsible for the pygmy phenotype, a mouse null mutant of Hmgi-c was produced by targeted disruption. Mouse embryonic stem (ES) cells were generated that had 3.0 kb of the Hmgi-c gene, encompassing exons 1 and 2, replaced with a neomycin-resistance gene (Fig. 3a). Matings between mice heterozygous for the mutated allele produced mice homozygous for the disrupted allele (Fig. 3b) at the expected mendelian frequency of approximately 25% (13 of 51). Immunoblot analysis demonstrated an absence of HMGI-C in protein extracts from homozygous embryos (Fig. 3c). Homozygous $Hmgi-c^{-/-}$ mice revealed the classical features of the pygmy phenotype, including reduced birth weight, craniofacial defects (shortened head), and an adult body weight of approximately 40%

(39.8 ± 2.9) of that of wild-type littermates[19]. It can therefore be concluded that absence of Hmgi-c expression in mice results in the pygmy phenotype.

A restricted number of adult tissues had previously been analysed[6], and it was established that the endogenous expression of Hmgi-c could not be detected. Hence, many more tissues were examined to investigate the temporal and tissue-specific expression pattern of Hmgi-c. Within the sensitivity of northern blot analysis, Hmgi-c expression was not detected in 18 adult tissues (data not shown). However, expression of Hmgi-c was observed during mouse embryogenesis (Fig. 4A) as early as 10.5 days post-coitum (d.p.c.), but essentially disappeared by 15.5 d.p.c. Remarkably, the other family member, Hmgi (y), showed a similar endogenous expression pattern (Fig. 4A) with expression readily observed in 10.5–16.5 d.p.c. mouse embryos. The predominant expression of Hmgi-c and Hmgi(y) during embryogenesis suggests this architectural factor family functions mainly in mammalian development.
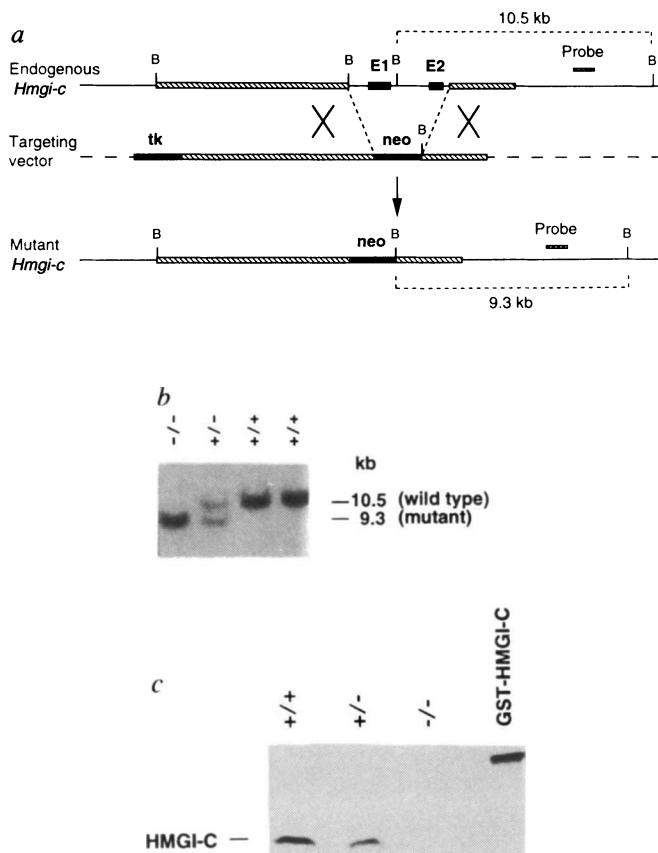

FIG. 3 Targeted disruption of the Hmgi-c gene. a, Targeting strategy. Endogenous Hmgi-c gene (top), targeting vector (middle) and predicted mutant gene (bottom). The targeting vector was created by replacing the 3-kb DNA fragment containing exon1 (E1) and exon2 (E2) with a PGK-neo cassette. The vector also includes a MC1-tk cassette at the 5′ end of the long homologous segment. B, BamHI; Probe, a 4-kb HincII fragment used to identify the disrupted allele. b, Southern blot analysis of mice from a heterozygous cross. DNA from tails of the mice was digested with BamHI and hybridized to the external probe (see a). The positions of the bands corresponding to the wild-type allele (10.5 kb) and the mutant allele (9.3 kb) are indicated. c, Western blot analysis of wild-type (+/+), heterozygous (+/−) and homozygous (−/−) 12.5 d.p.c. embryos with anti-GST-HMGI-C rabbit IgG.

METHODS. Genomic clones of the mouse Hmgi-c gene were isolated from the mouse pygmy locus as in Fig. 1. Linearized vector (10 μg) was electroporated into AB1 ES cells at 280 V, 500 μF, and homologous recombination events enriched for by selection with G418 (350 μg ml^{-1}) and 2 μM gangcyclovir (Syntex) on SNL76/7 feeder cells. Six targeted clones were obtained, and three were injected into C57BL/6J blastocysts to generate chimaeras. Chimaeric males were mated to C57BL/6J females, and heterozygous offspring intercrossed to produce subsequent generations. Southern blot analysis of the progeny from heterozygous crosses was performed as described[14]. Proteins were extracted from 12.5-d.p.c. mouse embryos from a heterozygous cross with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10% glycerol, 5 mM magnesium acetate, 0.2 mM EDTA, 1.0 mM PMSF and 1% SDS. Each sample (10 μg) was separated by 15% SDS-PAGE, transferred to a nylon membrane (Duralon, Stratagene), and HMGI-C was detected using rabbit IgG anti-mouse GST-HMGI-C, HRP-conjugated goat anti-rabbit IgG, and ECL substrate (Amersham).
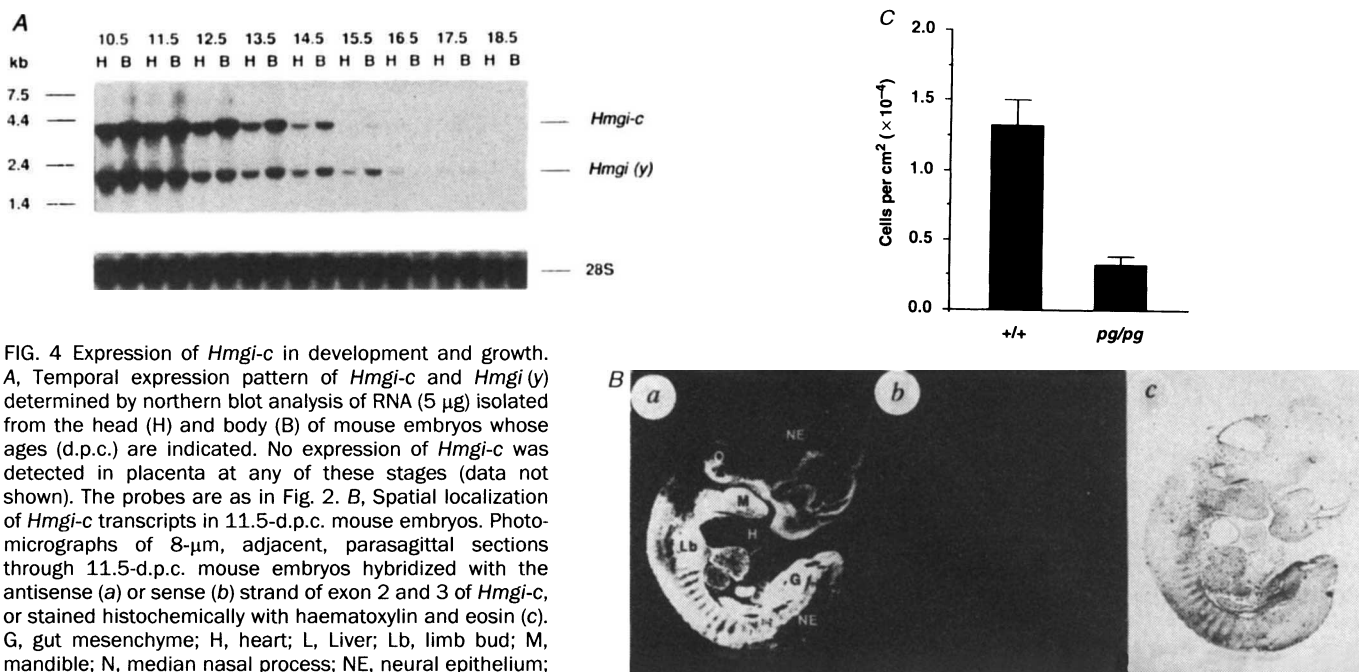
FIG. 4 Expression of *Hmgi-c* in development and growth. *A*, Temporal expression pattern of *Hmgi-c* and *Hmgi* (y) determined by northern blot analysis of RNA (5 μg) isolated from the head (H) and body (B) of mouse embryos whose ages (d.p.c.) are indicated. No expression of *Hmgi-c* was detected in placenta at any of these stages (data not shown). The probes are as in Fig. 2. *B*, Spatial localization of *Hmgi-c* transcripts in 11.5-d.p.c. mouse embryos. Photomicrographs of 8-μm, adjacent, parasagittal sections through 11.5-d.p.c. mouse embryos hybridized with the antisense (*a*) or sense (*b*) strand of exon 2 and 3 of *Hmgi-c*, or stained histochemically with haematoxylin and eosin (*c*). G, gut mesenchyme; H, heart; L, Liver; Lb, limb bud; M, mandible; N, median nasal process; NE, neural epithelium; O, otocyst. Magnification, ×15. *C*, Growth of wild-type and pygmy embryonic fibroblasts. Fibroblasts derived from 13.5-d.p.c. embryos were seeded at a concentration of 1.7×10^3 cells per cm^2 in DMEM containing 10% fetal bovine serum. Cell number (ordinate) was determined on day 4. Small bars represent standard deviations of triplicate experiments; $P < 0.001$. The genotypes of embryos were determined as previously described[12].

METHODS. For *in situ* hybridization, CBA/J embryos (11.5 d.p.c.) were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Paraffin sections were deparaffinized and hybridized with sense and antisense riboprobes corresponding to exons 2 and 3 of *Hmgi-c* as previously described[24]. Sections were stained with haematoxylin and eosin according to standard procedures.

The analysis of *Hmgi-c* expression was further extended by its localization in the normal developing mouse embryo. Expression was observed in most tissues and organs during embryogenesis, as exemplified by the 11.5-d.p.c. mouse embryo shown in Fig. 4*B*. Noticeably, *Hmgi-c* expression was not seen in the embryonic brain except in a small, localized region of the forebrain (Fig. 4*B*). This expression pattern coincides with previous studies which demonstrated that most tissues in pygmy mice were 40–50% smaller than wild-type tissues, the only tissue of normal size being the brain[19].

To investigate the role of *Hmgi-c* in cell growth, embryonic fibroblasts were cultured from homozygous and wild-type embryos. The number of *pg/pg* embryonic fibroblasts was fourfold less than wildtype fibroblasts after four days *in vitro* (Fig. 4*C*), and was not due to cell death. These results, along with those from other systems[20,21], are consistent with *Hmgi-c* being involved in cell proliferation and suggest that *Hmgi-c* functions in a cell-autonomous manner. Furthermore, absence of *Hmgi-c* expression in the pygmy mutant would then lead to a decrease in cell proliferation and result in the reduced size of all the tissues except for the brain.

Our results demonstrate that the absence of *Hmgi-c* causes growth retardation in pygmy mice. Although the precise molecular mechanism is not yet known, the function of HMGI proteins in cell proliferation could be regulated during the cell cycle through alteration of their DNA binding ability through phosphorylation by the cell cycle-dependent $p34^{cdc2}$ kinase[11]. Inactivation of the *Hmgi-c* gene would perturb the cell cycle in the developing embryo, and the resulting disruption of growth would produce the pygmy phenotype. The identification of the pygmy gene as *Hmgi-c* provides an insight into the control of mammalian growth and development, and aids the investigation of the biochemical nature of the African pygmy phenotype[4] and a multitude of growth hormone-resistant human dwarf syndromes[19]. □

1. Green, M. C. in *Genetic Variants and Strains of the Laboratory Mouse* (eds Lyon, M. & Searle, A.) 12–403 (Oxford Univ. Press, 1989).
2. Lin, S.-C. *et al. Nature* **364**, 208–213 (1993).
3. Li, S. *et al. Nature* **347**, 528–533 (1990).
4. Sinha, Y., Wolff, G., Baxter, S. & Domon, O. *Proc. Soc. exp. Biol. Med.* **162**, 221–223 (1979).
5. Nissley, S., Knazek, R. & Wolff, G. *Hormone Metab. Res.* **12**, 158–164 (1980).
6. Manfioletti, G. *et al. Nucleic Acids Res.* **19**, 6793–6797 (1991).
7. Grosschedl, R., Giese, K. & Pagel, J. *Trends Genet.* **10**, 94–100 (1994).
8. Saitoh, Y. & Laemmli, U. K. *Cell* **76**, 609–622 (1994).
9. Tjian, R. & Maniatis, T. *Cell* **77**, 5–8 (1994).
10. Johnson, K., Lehn, D., Elton, T., Barr, P. & Reeves, R. *J. biol. Chem.* **263**, 18338–18342 (1988).
11. Reeves, R., Langan, T. A. & Nissen, M. S. *Proc. natn. Acad. Sci. U.S.A.* **88**, 1671–1675 (1991).
12. Xiang, X., Benson, K. & Chada, K. *Science* **247**, 967–969 (1990).

13. Buckler, A. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 4005–4009 (1991).
14. Ausubel, F. *et al. Current Protocols in Molecular Biology* (Wiley, New York, 1988).
15. Thanos, D. & Maniatis, T. *Cell* **71**, 777–789 (1992).
16. Du, W., Thanos, D. & Maniatis, T. *Cell* **74**, 887–898 (1993).
17. King, J. *Genetics* **53**, 487–497 (1955).
18. Chirgwin, J., Przybyla, A., MacDonald, R. & Rutter, W. *Biochemistry* **18**, 5294–5299 (1979).
19. Benson, K. & Chada, K. *Genet. Res.* **64**, 27–33 (1994).
20. Ram, T., Reeves, R. & Hosick, H. L. *Cancer Res.* **53**, 2655–2660 (1993).
21. Berlingieri, M. T. *et al. Molec. cell. Biol.* **15**, 1545–1553 (1995).
22. Lehrach, H. *et al.* in *Genome Analysis Vol. 1. Genetic and Physical Mapping* (eds Davies, K. & Tilghman, S.) 39–81 (Cold Spring Harbor Laboratory Press, New York, 1990).
23. Barbu, V. & Dautry, F. *Nucleic Acids Res.* **17**, 7115 (1989).
24. Duncan, M., DiCicco-Bloom, E. M., Xiang, X., Benezra, R. & Chada, K. *Devl Biol.* **154**, 1–10 (1992).