Transfer of a yeast artificial chromosome carrying human DNA from *Saccharomyces cerevisiae* into mammalian cells

(gene transfer/cell fusion/cell culture)

VASSILIS PACHNIS, LARYSA PEVNY, RODNEY ROTHSTEIN, AND FRANK COSTANTINI

Department of Genetics and Development, Columbia University, 701 West 168th Street, New York, NY 10032

Communicated by Frank H. Ruddle, April 16, 1990

ABSTRACT To test the feasibility of transferring yeast artificial chromosomes (YACs) into mammalian cells, we modified a YAC that carries ≈ 450 kilobases (kb) of human DNA, by inserting a neomycin-resistance gene. *Saccharomyces cerevisiae* cells carrying this YAC were fused by polyethylene glycol to mouse L cells and G418-resistant colonies were obtained. A high percentage of these clones contained virtually intact YAC sequences as revealed by “*Alu* fingerprint” analysis and restriction enzyme analysis using pulsed-field gel electrophoresis. Furthermore, the YAC sequences were stably integrated into the mouse chromosomes, as shown by *in situ* hybridization and by the stability of the G418 resistance. These results establish that large segments of the mammalian genome, cloned in yeast, can be efficiently transferred into cultured mammalian cells.

The development of techniques for the transfer of cloned DNA into mammalian cells (calcium phosphate precipitation, protoplast fusion, electroporation, or microinjection) has played a pivotal role in understanding the control of gene expression and its role in various biological processes. The largest segment of cloned DNA that can be transferred into cells has been limited by the capacity of bacterial cloning vectors (plasmids, bacteriophages, and cosmids) and thus in most cases has not exceeded 50 kilobases (kb). Recently, however, yeast artificial chromosomes (YACs) have been used as cloning vehicles for the propagation in *Saccharomyces cerevisiae* of much larger segments of chromosomal DNA, hundreds of kilobases in length (1). The ability to introduce intact YACs back into cells in culture, or into embryos, would make feasible cellular transformation with large genes or gene complexes, as well as the rapid screening of large segments of the mammalian genome to identify genes that complement recessive mutations or confer dominant phenotypes.

We sought to examine the feasibility of transferring YAC DNA from *S. cerevisiae* into mammalian cells by cell fusion techniques that have been shown to result in the transfer of large continuous segments of chromosomal DNA (up to entire chromosomes) from one cell to another (e.g., generation of heterokaryons). In this report we establish that a YAC carrying ≈ 450 kb of human DNA and a neomycin-resistance gene can be transferred efficiently from *S. cerevisiae* cells into mouse fibroblasts (L cells) in culture by cell fusion. In a high percentage of cases the mouse cells contained virtually intact YAC DNA sequences that were stably integrated into the host chromosomes.

MATERIALS AND METHODS

Cells. Mouse L cells (a gift from Richard Axel, Columbia University) were maintained in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10% bovine calf serum (HyClone).

S. cerevisiae cells carrying the HY19 YAC (AB1380-HY19; AB1380: *MATa*, *ade2-1*, *can1-100*, *lys2-1*, *trp1*, *ura3*, *his5*, [*psi*+]) were a gift from Maynard Olson (Washington University, Saint Louis). Standard methods were used to propagate yeast (2). Yeast transformation was performed according to Burgers and Percival (3).

Fusion of L Cells with Yeast. In order to fuse L cells with the YAC-containing yeast AB1380-HY19-neo, 100-ml yeast cultures were grown to stationary phase in selection medium lacking uracil and tryptophan. Before fusion, yeast cultures were diluted 4-fold with rich medium (YPD) and incubated at 30°C for a further 45 min. The cells were subsequently collected by centrifugation, washed twice with sterile water, and resuspended in 15 ml of 1 M sorbitol/20 mM Tris-HCl, pH 7.5/10 mM EDTA/14 mM 2-mercaptoethanol. Three milligrams of Zymolyase 100T (Miles) was added and the yeast cells were incubated at 37°C for 15 min or until >95% became spheroplasts. Spheroplasts were pelleted and washed three times with 1 M sorbitol/20 mM Tris-HCl, pH 7.5. During the spheroplast washes, exponentially growing L cells were washed three times in serum-free DMEM and centrifuged onto the spheroplast pellet. The two pellets were mixed in 50 μ l of serum-free DMEM, and 500 μ l of 50% polyethylene glycol 1500 (Boehringer Mannheim) containing 50 μ M 2-mercaptoethanol, 5 mM CaCl_2 , and 5% dimethyl sulfoxide was added. After 2 min at room temperature the fused cells were diluted slowly by the addition of 5 ml of serum-free DMEM, incubated at room temperature for 30 min, and plated in DMEM containing 10% bovine calf serum. The neomycin analogue G418 (600 μ g/ml) was added 2 days later and cells were fed every 3–4 days until colonies were picked 2 weeks later.

Purification of Nucleic Acids. Genomic DNA was extracted from L cells according to the following protocol. The cells of a T-75 tissue culture flask were trypsinized, washed in phosphate-buffered saline, and collected by centrifugation. The cell pellet was resuspended in 1 ml of 0.1 M NaCl/0.2 M sucrose/50 mM EDTA/30 mM Tris-HCl, pH 8.0, and were subsequently lysed with 0.25 ml of 0.25 M EDTA/0.5 M Tris-HCl, pH 9.2/2.5% SDS. The lysate was incubated at 52°C for 15 min and then digested with proteinase K (4 mg/ml) at 37°C for 2 hr. After the addition of 1.25 ml of 10 mM Tris-HCl, pH 8.0/1 M NaCl, extraction with phenol/chloroform (1:1, vol/vol), and ethanol precipitation, the DNA was resuspended in 200 μ l of 10 mM Tris-HCl, pH 7.5/1 mM EDTA. To isolate yeast DNA we followed the protocol described in ref. 4.

Standard methods were used for preparation of high molecular weight DNA from L cells and yeast spheroplasts and for pulsed-field gel electrophoresis (PFGE) (5).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: YAC, yeast artificial chromosome; PFGE, pulsed-field gel electrophoresis; G418^r, G418-resistant.

In Situ Hybridization. For *in situ* hybridization to metaphase spreads of G418-resistant (G418^r) L-cell clones, we followed the protocol of Lichter *et al.* (6).

RESULTS

Modification of the HY19 YAC. We transferred the YAC HY19, which carries ≈ 450 kb of DNA from an unidentified human locus, into mouse L cells by generating transient hybrids between the L cells and spheroplasts of the host *S. cerevisiae* strain (AB1380-HY19-neo). This approach was based (i) on the ability of a yeast multicopy plasmid carrying a neomycin-resistance (*neo*) gene to be transferred with high frequency to mammalian cells by spheroplast fusion (ref. 7 and unpublished data) and (ii) on the demonstration that an intact chromosome from the fission yeast *Schizosaccharomyces pombe* could be transferred into C127 mouse cells by similar techniques (8). To allow for selection of L cells that acquired the human DNA sequences, we modified the HY19 YAC by inserting the MC1POLA (*neo*) gene, which had been shown to confer G418 resistance on mammalian cells (9).

The strategy for this modification (Fig. 1A) entailed the use of a YAC-derived fragment of human DNA as vector for targeting the *neo* gene, coupled to a yeast selectable marker, *ADE2* (the *ADE2* gene confers adenine prototrophy on *ade2* yeast strains; Jeff Lemontt, personal communication), into the HY19 chromosome by homologous recombination (11). More specifically, the 4.0-kb *Sac* I fragment from the HY19 chromosome (Fig. 1A *Top*) was derived from a recombinant λ clone that was identified as carrying human DNA by screening an AB1380-HY19 genomic library with a human *Alu* repetitive probe (blur-8; ref. 12). This fragment was subsequently modified by substituting the 3.2-kb *Bam*HI fragment, which contains the MC1POLA (*neo*) and *ADE2* genes, for the internal 0.6-kb *Bam*HI fragment. AB1380-HY19 cells were transformed with the *Sac*-*ADE2*-*neo* fragment (Fig. 1A *Middle*). Some of the resulting Ade⁺ transformants should have replaced, by homologous recombination, the endogenous wild-type *Sac* I fragment with the modified *Sac*-*ADE2*-*neo* fragment. The *neo* and *ADE2* genes were shown to have integrated into the endogenous *Sac* I fragment

in 1 out of 10 Ade⁺ transformants analyzed (AB1380-HY19-neo). This was based on two criteria: (i) restriction enzyme mapping of the integration site of *Sac*-*ADE2*-*neo* and (ii) the deletion of the internal 0.6-kb *Bam*HI fragment from the HY19 chromosome. Further analysis revealed that AB1380-HY19-neo had acquired multiple copies (20 copies) of the *Sac*-*ADE2*-*neo* fragment in a head-to-tail arrangement (data not shown). [Subsequent to our experiments we became aware that the particular *ADE2* gene we used to transform the parental yeast cells functions poorly in *S. cerevisiae* cells (unpublished data). This fact may provide an explanation for the high copy number of the *Sac*-*ADE2*-*neo* fragment integrated in the HY19-neo YAC; a single copy would be insufficient to confer adenine prototrophy on the yeast cells.]

To verify that the *neo* sequences had integrated on the HY19 YAC, chromosomes from the parental AB1380-HY19 strain and the derivative AB1380-HY19-neo strain were resolved by PFGE (13, 14) and hybridized to probes for the *neo* gene or the *Alu* repeat sequences. The chromosomes carrying the *Alu* (human DNA) and *neo* sequences comigrated (Fig. 1B), suggesting that the *neo* gene is physically linked to the human DNA on the HY19 YAC. Note that the modified HY19-neo chromosome is larger (600 kb) than the original HY19 chromosome (450 kb). This size difference can be accounted for by the amount of additional sequences (*ADE2* and *neo*) integrated on the HY19 chromosome. The integration of the exogenous sequences on the HY19 chromosome by the strategy depicted in Fig. 1A establishes that human DNA propagated in YAC vectors can be modified by homologous recombination in *S. cerevisiae*.

Generation of G418^r L-Cell Clones by Fusion to AB1380-HY19-neo *S. cerevisiae* Cells. To fuse yeast carrying the HY19-neo chromosome to mouse L cells, we used conditions similar to those of Allshire *et al.* (8). Spheroplasts, generated by digesting the yeast cell wall with Zymolyase, were fused to L cells in suspension in the presence of polyethylene glycol 1500. The cells were subsequently transferred to fresh medium, cultured for 2 days, and then grown in the presence of G418 (600 μ g/ml). After 10–15 days in culture, ≈ 50 G418^r L-cell colonies appeared for every 10^6 cells used for fusion. The presence of the *neo* gene in these G418^r colonies was

FIG. 1. Integration of the MC1POLA (*neo*) gene into the HY19 chromosome. (A) (*Top*) Diagram of the original HY19 chromosome. The telomeres (*TEL*), centromere (*CEN*), yeast autonomous replication sequence (*ARS*), and the two yeast selectable markers (*TRP1* and *URA3*) on the HY19 YAC are shown. (*Middle*) The two selectable markers (*ADE2* and *neo*) present on the *Sac*-*ADE2*-*neo* fragment. (*Bottom*) Part of the modified YAC (HY19-neo). B, *Bam*HI. Solid bars indicate human DNA. (B) PFGE analysis of HY19 and HY19-neo chromosomes. Chromosomal DNA was prepared in agarose plugs from AB1380-HY19 (lanes 1 and 3) and AB1380-HY19-neo (lanes 2 and 4) cells and separated, undigested, on a contour-clamped homogeneous electric field (CHEF) gel (10). DNA was transferred to a nitrocellulose membrane and hybridized with an *Alu* repeat (lanes 1 and 2) or the *neo* (lanes 3 and 4) probe.

confirmed by Southern blot analysis. The frequency of G418^r colonies was identical to that observed when L cells were fused with AB1380 yeast containing a multicopy plasmid carrying the *neo* gene (unpublished data), indicating that the transfer of YAC sequences is no less efficient than the transfer of plasmid sequences. In control experiments, fusion of L cells with spheroplasts of the parental yeast strain AB1380 never resulted in G418^r cells.

A High Percentage of G418^r L-Cell Clones Contain a Largely Intact Copy of the HY19-neo YAC. To determine the extent of HY19 human DNA sequences cotransferred into the G418^r L cells, DNA from the first eight independent G418^r L-cell clones (YF3-1, -4, -6, -8, -10, -11, -26, and -27) was compared with the intact HY19-neo chromosome present in yeast, by restriction enzyme digestion and Southern blot analysis. Seven of these clones contained restriction enzyme fragments that hybridized to the *Alu* probe, while the eighth (YF3-1) showed no evidence of human DNA sequences (Fig. 2). More important, four of the L-cell clones (YF3-6, -10, -26, and -27) generated a set of bands ("Alu fingerprint") virtually indistinguishable from that of donor HY19-neo DNA, with each of the four restriction enzymes used for this analysis (*EcoRI*, *HindIII*, *BamHI*, and *PstI*; digests with two of these enzymes are shown in Fig. 2). Assuming that the *Alu* repeat elements are dispersed throughout the human DNA present on HY19, this suggests that the majority of the HY19-neo chromosome was transferred into transformants YF3-6, -10, -26, and -27. Furthermore, the absence of any novel restriction enzyme fragments hybridizing to the *Alu* probe suggests that the human DNA sequences have undergone no major rearrangements upon transfer into the mouse cells. In contrast, three clones (YF3-4, -8, and -11) contained only a subset of the *Alu*-positive restriction fragments, indicating that these clones acquired only a portion of the HY19-neo chromosome. In a total of 19 G418^r clones examined, 9 (47%) had acquired the entire set of *Alu*-positive restriction enzyme fragments present in HY19-neo, 7 (37%) had acquired only a subset, and 3 (16%) contained no *Alu*-positive DNA even though they had acquired the *neo* sequences.

Our subsequent analysis focused on several of the L-cell transformants that displayed the complete *Alu* fingerprint of the HY19-neo chromosome. First, to establish further that no gross rearrangements of the human DNA had occurred upon transfer into the L cells, we examined the sizes of fragments generated by digestion with two rare-cutting restriction endonucleases. When AB1380-HY19-neo DNA was digested with *XhoI* or *ClaI* (Fig. 3), unique restriction fragments of ≈ 350 kb hybridized with the *neo* probe. DNA fragments of the identical sizes were observed in digests of DNA from L-cell clones YF3-10, -26, and -27, strongly suggesting that these restriction fragments were transferred into those clones intact. In a fourth clone, YF3-6, slightly smaller fragments were observed with both enzymes. Further restriction mapping of the HY19 sequences in this clone established that the size difference was localized in the tandem array of the *Sac*-*ADE2*-*neo* DNA fragments (data not shown). This suggests that the shift in mobility of the *neo*-containing *ClaI* and *XhoI* fragments in YF3-6 cells is due to excision of several *Sac*-*ADE2*-*neo* copies by intrachromosomal recombination. We have been unable to determine whether such a recombination event took place in the yeast cells before fusion or, alternatively, in the L cells, since intrachromosomal recombination can occur in both types of cells (15, 16).

Next, to determine whether subtelomeric YAC vector sequences from the HY19-neo chromosome were present in the L-cell transformants that displayed a complete *Alu* fingerprint, DNA from YF3-6, -10, -26, and -27 cells was hybridized with *URA3* and *TRP1* probes that define the left and right YAC arms, respectively (ref. 1 and Fig. 1A). DNA from YF3-6 cells was positive with both probes, while DNAs from the other three clones were negative with both probes (data not shown). Together with the data described above, this suggests that clone YF3-6 has acquired the entire human DNA segment present on the HY19 chromosome as well as the YAC arms. On the other hand, clones YF3-10, -26, and -27 have acquired the majority of the human sequences, as determined by the complete *Alu* fingerprint, but have lost both ends of the YAC.

FIG. 2. *Alu* fingerprint analysis of human DNA in L-cell transformants. DNA from AB1380-HY19-neo cells (150 ng), the parental L cells (10 μ g), and eight YF3 clones (10 μ g) was digested with *EcoRI* or *HindIII* and electrophoresed in a 1% agarose gel. The DNA was subsequently transferred to a nitrocellulose membrane and hybridized to a human *Alu* repeat probe.

FIG. 3. PFGE analysis of the HY19-neo DNA in four G418^r L-cell transformants. Undigested DNA from AB1380-HY19-neo cells (first and last lanes) was electrophoresed in parallel with *Xho* I- or *Cla* I-digested DNA from AB1380-HY19-neo and YF3-6, -10, -26, and -27 cells. The DNA was subsequently transferred to a nitrocellulose membrane and hybridized with a *neo* probe.

The HY19-neo YAC Has Been Stably Integrated into L-Cell Chromosomes. Allshire *et al.* (8) showed that an intact chromosome from *Sch. pombe* can function as an unstable, extrachromosomal, autonomously replicating element in mouse cells. It was therefore of interest to determine the status of the essentially intact HY19 chromosome in YF3-6 cells, as well as that of the HY19 sequences in the other L-cell transformants. For this purpose, metaphase chromosome spreads were prepared from clones YF3-6, -26, and -27 and

hybridized *in situ* to biotinylated *Sac-ADE2-neo* DNA. Following incubation with fluorescein-conjugated avidin, the hybridization signal was visualized by fluorescence microscopy (6). A single site of hybridization, always associated with a unique mouse chromosome, was observed in each cell line examined. An example of such a hybridization experiment is shown in Fig. 4.

As an independent test for chromosomal integration of the *neo* gene, the L-cell clones were grown in the absence of

FIG. 4. Detection of YAC DNA in a mouse chromosome of clone YF3-27 by *in situ* hybridization. (A) Photomicrograph of a representative metaphase spread from YF3-27 cells hybridized to biotinylated *Sac-ADE2-neo* fragment. The hybridized probe was detected with fluorescein-conjugated avidin and fluorescence microscopy. (B) The same metaphase spread counterstained with 4',6-diamidino-2-phenylindole.

G418 for seven passages (30–40 generations) and then tested for their relative plating efficiency in the presence or absence of G418. Clones YF3-10, -26, and -27 displayed complete stability of G418 resistance by this assay, consistent with the observed integration of HY19-neo sequences into mouse chromosomes. In contrast, YF3-6 cells displayed a dramatic loss of G418 resistance (3% relative plating efficiency) when grown in the absence of selection, despite the apparent chromosomal integration of the HY19-neo sequences in these cells. Consistent with the *in situ* hybridization results, the extinction of the G418^r phenotype in YF3-6 cells was not associated with loss of the neo sequences, as determined by Southern blot analysis. Therefore, the instability of G418 resistance in this clone is not due to the autonomous, extrachromosomal propagation of the HY19-neo sequences but more likely due to gradual extinction of expression of the neo gene in this particular clone. Although the mechanism of inactivation of the neo gene in YF3-6 cells is unknown, it may involve methylation of the HY19-neo sequences. *De novo* methylation, accompanied by loss of expression, has been observed for genes transfected into mouse L cells by calcium phosphate precipitation (17–19).

DISCUSSION

We have shown that the fusion of *S. cerevisiae* spheroplasts with mammalian cells permits high-frequency transfer of a YAC containing 600 kb of human DNA from yeast into the mammalian nucleus. In ~50% of such transformants, the entire complement of the human cloned sequences and the neo gene, as determined by *Alu* I fingerprint analysis, have been stably integrated into the mouse chromosomes without any apparent rearrangement.

As the human DNA on the YAC we employed has not been characterized, we were unable to test for expression of any human genes transferred on the YAC. However, the neo gene, which utilizes a mammalian promoter and enhancer (9), was clearly expressed in the L cells as this was the basis for their selection. Therefore our experiments show that passage of mammalian (YAC) DNA through *S. cerevisiae* does not result in the permanent inactivation of gene expression.

The ability to transfer large continuous segments of functional mammalian DNA from yeast cells back into mammalian cells should make feasible expression studies with very large genes (e.g., factor VIII or even dystrophin), gene complexes (e.g., immunoglobulin or major histocompatibility complex), or genes with distant regulatory elements (e.g., globin genes). Furthermore, specific mutations can be introduced onto the cloned YAC DNA in yeast cells by using the well developed genetic manipulation techniques available for *S. cerevisiae*. The effects of such mutations could subsequently be tested upon transfer of the modified YAC DNA back into mammalian cells.

The application of this YAC transfer system to pluripotent embryonic stem cells (20, 21), which are capable of colonizing the somatic as well as the germ cell lineages when implanted into early mouse embryos (22), could lead to the generation of transgenic animals that carry and transmit any YAC. This would permit the relatively rapid screening of large segments of mouse or human chromosomes for loci that can complement existing mouse mutations or confer dominant mutant or disease phenotypes. Such applications will require that a selectable marker be introduced into each YAC to be tested, without disrupting the gene(s) of interest. While in the present study we have inserted the neo gene into the

human DNA of the YAC, it should be equally feasible to insert a selectable marker into a YAC vector arm by homologous recombination. Furthermore, the insertion of a different selectable marker into each of the two YAC arms may serve to increase the frequency of transfer of intact YACs into the mammalian cells.

Finally, among the L-cell clones we have examined we have been unable to identify any that carry the HY19-neo YAC as an autonomously replicating extrachromosomal element. Presumably structural elements necessary for the function of a mammalian chromosome are missing from the HY19-neo YAC. Such a YAC could serve as a framework for the addition and testing of structural elements (e.g., centromeres) derived from the mammalian genome that could constitute a stable autonomously replicating unit in mammalian cells.

We thank Maynard Olson (Washington University, Saint Louis) for the gift of HY19 YAC and William Klein for critically reviewing the manuscript. This work was supported by grants from the National Institutes of Health to F.C. (HD17704 and CA22376) and to R.R. (GM34587). V.P. is a Special Fellow of the Leukemia Society, R.R. is an American Heart Association Established Investigator, and F.C. is a Pew Scholar in the Biomedical Sciences.

- Burke, D., Carle, G. F. & Olson, M. V. (1987) *Science* **236**, 806–812.
- Sherman, F., Fink, G. R. & Hicks, J. B. (1986) *Laboratory Course Manual for Methods in Yeast Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
- Burgers, P. M. & Percival, K. J. (1987) *Anal. Biochem.* **166**, 391–397.
- Rothstein, R. J. (1985) in *DNA Cloning: A Practical Approach*, ed. Glover, D. M. (IRL, Oxford), pp. 45–66.
- Smith, C. L., Klco, S. R. & Cantor, C. R. (1988) in *Genome Analysis: A Practical Approach*, ed. Davies, K. E. (IRL, Oxford), pp. 41–72.
- Lichter, P., Cremer, T., Borden, J., Manuelidis, L. & Ward, D. C. (1988) *Hum. Genet.* **80**, 224–234.
- Ward, M., Scott, R. J., Davey, M. R., Clothier, R. H., Cocking, E. C. & Balls, M. (1986) *Somatic Cell Mol. Genet.* **12**, 101–109.
- Allshire, R. C., Cranston, G., Gosden, J. R., Maule, J. C., Hastie, N. D. & Fantes, P. A. (1987) *Cell* **50**, 391–403.
- Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51**, 503–512.
- Chu, G., Volrath, D. & Davis, R. W. (1986) *Science* **234**, 1582–1585.
- Rothstein, R. J. (1983) *Methods Enzymol.* **101**, 202–211.
- Jelinek, W. R., Toomey, T. P., Leinwand, L., Duncan, C. H., Biro, P. A., Choudary, P. V., Weissman, S. M., Rubin, C. M., Houck, C. M., Deininger, P. L. & Schmid, C. D. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1398–1402.
- Schwartz, D. & Cantor, C. R. (1984) *Cell* **37**, 67–75.
- Carle, G. F. & Olson, M. V. (1984) *Nucleic Acids Res.* **12**, 5647–5664.
- Szostak, J. W. & Wu, R. (1980) *Nature (London)* **284**, 426–430.
- Liskay, R. M., Stachelek, J. L. & Letsou, A. (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49**, 183–189.
- Pollack, Y., Stein, R., Razin, A. & Cedar, H. (1984) *Proc. Natl. Acad. Sci. USA* **77**, 6463–6467.
- Desrosiers, R. C., Mulder, C. & Fleckenstein, B. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3839–3843.
- Sutter, D. & Doerfler, W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 253–256.
- Evans, M. J. & Kaufman, M. H. (1981) *Nature (London)* **292**, 154–156.
- Martin, G. R. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638.
- Bradley, A., Evans, M., Kaufman, M. H. & Robertson, E. (1984) *Nature (London)* **309**, 255–256.