Requirement for *Xist* in X chromosome inactivation

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The *Xist* gene has been proposed as a candidate for the X inactivation centre, the master regulatory switch locus that controls X chromosome inactivation. So far this hypothesis has been supported solely by indirect evidence. Here we describe gene targeting of *Xist*, and provide evidence for its absolute requirement in the process of X chromosome inactivation.

DOSAGE compensation of X-linked genes in mammals is achieved by the transcriptional silencing of a single X chromosome early in the development of the female embryo. This process, termed X chromosome inactivation (X inactivation), is normally random, with an equal probability that either X chromosome will be inactivated in a given cell[1]. Recent progress towards understanding X inactivation has been made through attempts to identify the X inactivation centre (Xic), a *cis*-acting locus on the X chromosome which is the master regulatory switch controlling this process[2].

Initiation, the first step in X inactivation, involves the cellular determination of how many (if any) and which X chromosomes to inactivate. Experimental observations on X; autosome translocations and X chromosome deletions suggest that the Xic is required for the initiation of X inactivation in early embryogenesis, there being an absolute requirement for at least two Xics to be present[3,4]. Further evidence supporting a role for the Xic in the initiation of X inactivation comes from studies of alleles at the X controlling element (*Xce*) locus, which maps to the Xic region on the mouse X chromosome[5–7]. *Xce* homozygotes undergo normal, random X inactivation, whereas heterozygotes show primary non-random X inactivation[8], as there is a greater probability that an X chromosome bearing a strong *Xce* allele will remain active. Thus the Xic is implicated both in the decision as to how many X chromosomes are inactivated (counting), and also which X chromosomes are inactivated (choice). Studies on X-chromosome rearrangements have also demonstrated that the Xic is required *in cis* for the spread of inactivation (propagation) to occur along the entire X chromosome[9,10]. After the inactive state has been established, X inactivation patterns are stably mantained in all subsequent cell divisions. The inactive state is stably maintained after the loss of the entire minimal XIC region on the human X chromosome[11], which suggests that, although the Xic is required for initiation and propagation, it is not required for the maintenance of X inactivation.

A candidate for the Xic, termed *XIST* (X-inactive specific transcript), has been identified within the human minimum XIC interval[12,13]. In contrast to all other X-linked genes, *XIST* is expressed exclusively from the inactive X chromosome. The equivalent mouse gene (*Xist*) was cloned through homology, localized to the mouse Xic interval, and shown to be expressed exclusively from the inactive X chromosome[14,15]. The *XIST/Xist* gene encodes a large RNA transcript which lacks protein coding potential and is localized in the nucleus, apparently in direct association with the inactive X chromosome[16,17]. This finding has led to the proposal that the transcript acts *in cis* to propagate X inactivation. Alternative models remain tenable, for example that the *XIST/Xist* locus represents a nucleation centre for heterochromatin induction that is initiated by transcription of the locus[16]. Studies of expression of the mouse gene in early embryogenesis[18,19], and in XX embryonic stem (ES) cells[18], which undergo X inactivation on differentiation *in vitro*[3], demonstrate that *Xist* expression precedes overt X inactivation, consistent with it having a role in the initiation process. *Xist* is not expressed in male tissues other than male germ cells just before meiosis[18,20,21]. This observation has led to the suggestion that *Xist* could also be involved in the transcriptional silencing of the XY bivalent in the sex vesicle.

We have tested directly the hypothesis that *Xist* is required for X inactivation, by introducing a targeted deletion into a single *Xist* allele in an XX embryonic stem (ES) cell line, and have investigated the effect of this mutation on X inactivation following differentiation of mutant cells *in vitro* and *in vivo*. At the outset we predicted three possible outcomes: (1) that mutant cells would fail to register the presence of two Xics, and would entirely fail to undergo X inactivation; (2) that the mutation would disrupt X inactivation such that the targeted X chromosome would fail to X inactivate, although the normal X chromosome would undergo X inactivation; and (3) that the mutation would have no effect on X inactivation at all. Our results show that the second prediction is correct, and that X inactivation fails to occur *in cis* on the X chromosome bearing the deleted *Xist* allele.

XX ES cells: an *in vitro* model

We targeted *Xist* in XX ES cells, as this allows analysis of the initiation of X inactivation by *in vitro* differentiation of mutant ES cells. We derived an XX ES cell line (PGK12.1) using delayed blastocysts from a 129 female crossed to a (PGK × 129)F_1 male. The PGK12.1 cell line is thus heterozygous for a PGK and a 129 derived X chromosome. Because the X chromosome in the C3H/He-Pgk-1^a/Ws (PGK) mouse is derived from a wild mouse strain[22], it is relatively easy to detect polymorphisms for X-linked markers to assist in subsequent analysis.

We have previously shown that the PGK12.1 ES line expresses both *Xist* alleles and undergoes random X inactivation upon differentiation *in vitro*[23]. However, analysis of the methylation status of several CpG sites at the 5′ end of the *Xist* gene indicated that undifferentiated XX ES cells are methylated at approximately 50% of the level seen in XY ES cells, which show near-complete methylation. This observation led us to hypothesize that only a single *Xist* allele is methylated in an individual XX ES cell, and that this may reflect predetermination with respect to *Xist* expression and X inactivation[23]. As predetermination of individual ES cells would have important implications in terms of interpreting the outcome of *Xist* gene targeting, we have investi-

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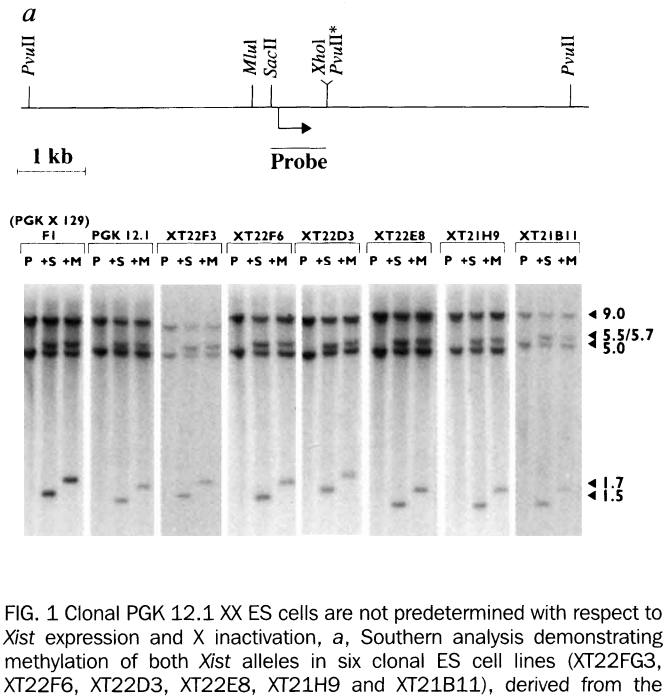

FIG. 1 Clonal PGK 12.1 XX ES cells are not predetermined with respect to *Xist* expression and X inactivation, *a*, Southern analysis demonstrating methylation of both *Xist* alleles in six clonal ES cell lines (XT22FG3, XT22F6, XT22D3, XT22E8, XT21H9 and XT21B11), derived from the PGK12.1 ES cell line. A polymorphic *Pvu*II site, indicated on the map by an asterisk, was used to distinguish the PGK (5 kb), and 129 (9 kb) alleles. The transcription start site is indicated with an arrow. After digestion with *Pvu*II (P), samples were digested with the methylation-sensitive restriction enzymes *Sac*II (+S) or *Mlu*I (+M). The results indicate approximately 50% methylation of both alleles in all six clonal cell lines. Controls were (PGK × 129)F_1 female mouse and non-clonal PGK12.1 ES cells. The probe used was the *Sac*II/*Xho*I fragment indicated. Band sizes are marked in kilobases. *b*, The relative RNA levels of *Xist* and *Pgk-1* alleles (A, PGK; B, 129) in differentiated clonal PGK12.1 lines was determined by quantitative SNuPE analysis. Quantification of SNuPE assays is expressed for the PGK allele as the percentage of total ±s.e.m. The results indicate random *Xist* expression and X inactivation with a pronounced *Xce* effect. Controls are adult tissue RNA (129 or PGK). Three independent cultures were assayed for each clonal cell line. Differentiation was monitored by slot-blot analysis of *Oct4* RNA levels. Quantification is expressed for differentiated cells (EB) as percentage of ES cell level ±s.e.m. Downregulation of *Oct4* was less pronounced with XT21B11 cells than with the other lines.

METHODS. ES cell line PGK12.1 was derived essentially as described previously[37], using delayed blastocysts from a $129^{ola/hsd}$ × (C3H/He – Pgk – 1^a/Ws × $129^{ola/hsd}$)F_1 backcross. ES cells were cultured in ES cell medium; DMEM with 4.5 g l^{-1} glucose, no sodium pyruvate, 2 mM glutamine, 1 × non-essential amino acids (GIBCO), 0.05 mg ml^{-1} streptomycin, 500 i.u. l^{-1} penicillin, 0.1 mM 2-mercaptoethanol, 20% fetal calf serum, and 10^3 ug ml^{-1} recombinant LIF(GIBCO), at 37 °C, 5% CO_2, 100% humidity. Clonal PGK12.1 lines were isolated by geneticin (400 μg ml^{-1}) selection following transfection with *Pgk-neo*. Methylation analysis was as described previously[23]. Cells were differentiated using the embryoid body outgrowth method[37]. Cells were first plated in EB medium: DMEM with 4.5 g l^{-1} glucose, no sodium pyruvate, 2 mM glutamine, 0.05 mg ml^{-1} streptomycin, 500 i.u. l^{-1} penicillin, 0.1 mM 2-mercaptoethanol, 10% fetal calf serum, at 37 °C, 5% CO_2, 100% humidity. After 3 days, cells were transferred to suspension culture for 3–6 days, and then returned to monolayer culture for a further 3 days before collection. RNA was prepared using RNAzol reagent (Biogenesis). DNA isolation, Southern and northern slot-blot analysis were performed using standard methods. Slot blots were loaded with 5 μg RNA, and probed with an *Oct4* cDNA probe corresponding to bases 491–953 of the published sequence[38]. Quantitative SNuPE analysis was performed as described previously[24]. *Pgk-1* SNuPE analysis used primers and conditions described previously[24]. For *Xist*, RT–PCR was as described previously[18]. SNuPE, 1 cycle of 94 °C 1′, 50 °C 2′ and 72 °C 1′, was performed using primer Xist-9974; GGTTCTCTCCAGAAGCTAGGA, which detects a previously described polymorphism[20] (129, A; PGK, G). Quantification of SNuPE and *Oct4* RNA slot blots was performed on a Molecular Dynamics phosphorimager. All SNuPE results were normalized using RNA samples with known allelic levels[24].

gated this further by analysing six clonal PGK12.1 ES cell lines. If individual ES cells are predetermined, in clonal lines we would expect to see methylation of only one allele and non-random *Xist* expression and X inactivation following differentiation. Methylation analysis of *Xist* in the clonal cell lines is illustrated in Fig. 1*a*; both alleles show approximately 50% methylation levels. Expression of *Xist* and *Pgk-1* in differentiated cells was analysed using the quantitative allele-specific single nucleotide primer extension assay (SNuPE)[24–26] (Fig. 1*b*). In this experiment the extent of differentiation was assessed by monitoring downregulation of *Oct4* RNA, an early marker of ES cell differentiation[27] (Fib. 1*b*). In each clone we observed expression of the 129 strain (B) and PGK strain (A) alleles of *Xist* and *Pgk-1*. Quantification of these results demonstrated lower levels of PGK strain *Xist* RNA and higher levels of PGK strain *Pgk-1* RNA, consistent with the PGK X chromosome carrying a stronger *Xce* allele (Xce^c) than the 129 X chromosome (Xce^a). This result accords with previous observations of an *Xce* effect in similar XX ES cell lines[26]. These results demonstrate that clonal PGK12.1 cells are not pre-determined with respect to *Xist* expression or X inactivation.

Targeted mutagenesis

The gene-targeting strategy adopted is illustrated in Fig. 2. Because *Xist* RNA does not encode a protein, we created a large deletion of the *Xist* gene to maximize the probability of creating a null allele. The targeting construct, pGPT15, was assembled using 5′ and 3′ homologous fragments separated by approximately 7 kilobases (kb) of sequence from exon 1 of the *Xist* gene and including 36 base pairs (bp) of *Xist* minimal promoter sequence. The construct (Fig. 2*a*) includes a *Pgk-neo* gene for positive selection of stable integrants, and the herpes simplex virus thymidine kinase gene (HSV-TK) for selection against non-homologous events[28]. We screened 2,588 stably transfected colonies by Southern analysis, and a single homologous integrant,

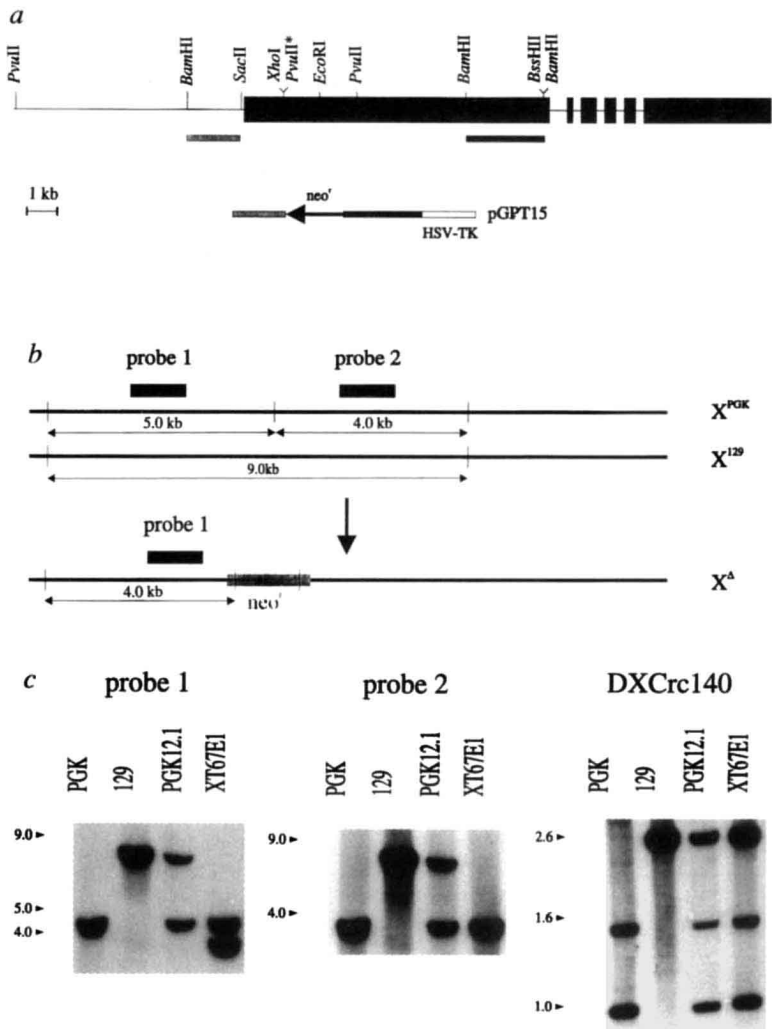

FIG. 2 Targeted deletion of the 129 strain *Xist* allele in PGK12.1 ES cells. *a*, A partial restriction map of the *Xist* locus indicating the gene structure and regions of homology used to construct the targeting vector pGPT15. *b*, A *Pvu*II polymorphism was used to distinguish *Xist* alleles in PGK12.1 cells. The size of expected restriction fragments detected with probes 1 and 2 in the parent and targeted 129 loci are shown. *c*, Southern blot analysis of *Pvu*II-digested control female tissue DNA (PGK and 129 strain), and PGK12.1 and XT67E1 ES cell line DNA, using probes 1 and 2, shows that the cell line XT67E1 carries a targeted deletion of the 129 strain *Xist* locus (sizes in kilobases). Probe 1 detects a 4-kb band, as predicted for homologous integration, and the 5-kb PGK-derived band indicating that the 129 allele has been targeted. Probe 2, which lies within the deleted region, only detects the 5-kb PGK allele. A *Pvu*II polymorphism at the *DXCrc140* locus[39] was used to ascertain that XT67E1 cells had not eliminated the PGK strain X chromosome.
METHODS. The 5′ (*Bam*H1/*Sac*II) and 3′(*Bam*H1/*Bam*H1) homologous fragments used in the pGPT15 construct were isolated from genomic clones from a 129 library in lambda DASH (Stratagene). PGK12.1 ES cells (1×10^7–2×10^7) were electroporated with 20 μg of linearized pGPT15 (500 V/25 μF in a Biorad Gene Pulser), and selection was then performed over 7 days in ES cell medium containing 400 μg ml^{-1} geneticin and 2 μM gancyclovir. Individual colonies were picked onto 96-well plates. Replicate plates were prepared for isolation of DNA and Southern analysis as described previously[40]. Southern blots were probed with radiolabelled *Xist* probes as indicated, and also with the pEM140A probe from the *DXCrc140* locus[39].

XT67E1, was identified. Southern analysis, using a *Pvu*II polymorphism to distinguish the 129 and PGK *Xist* alleles (Fig. 2*b*), demonstrated that the targeting event occurred on the 129-derived X chromosome (Fig. 2*c*). Further analysis with a 3′ probe, and also with a range of restriction enzymes, was used to confirm this result and demonstrate correct homologous integration (not shown).

X inactivation *in vitro*

Xist expression was analysed in differentiated derivatives of XT67E1 cells, using a *Hin*dIII polymorphism that allowed transcripts from the two alleles to be distinguished[18]. As expected, differentiated XT67E1 cells only expressed *Xist* from the non-targeted allele (Fig. 3*a*), confirming that the deletion of the 129 *Xist* locus results in a null allele. This finding also suggests that the counting function of the Xic is not disrupted in targeted cells, that is, XT67E1 cells retain the ability to register the presence of two Xics. However, disruption of the counting mechanism cannot be precluded from this result alone as it is not known formally that *Xist* expression is an absolute indicator of the decision to undergo X inactivation. For this reason, differentiated cells were analysed for the presence of an inactive X chromosome by using a cytogenetic late-replication assay[29–31]. Metaphase spreads were prepared from control female BALB/c cultured thymocytes, and from PGK12.1 and XT67E1 ES cells and their differentiated derivatives, and were assayed for the presence of a late-replicating X chromosome in a double-blind screen (Fig. 3*b*). Late-replicating X chromosomes were not found in undifferentiated ES cells, consistent with there being two active X chromosomes, but were observed in both PGK12.1 and XT67E1 differentiated cells. This result confirms that the *Xist* deletion does not result in loss of the counting function of the Xic. We did, however, observe a slightly reduced frequency of late-replicating X chromosomes in differentiated XT67E1 (29%) compared with PGK12.1 cells (44%) (see discussion).

Nonrandom X inactivation *in vitro*

To determine whether targeted cells undergo normal random X inactivation, we used the SNuPE assay to assess the relative levels of X-linked gene products in PGK12.1 and XT67E1 ES cells and their differentiated derivatives. In addition to the precharacterized polymorphism used to assess the relative expression level of the two alleles at the *Pgk-1* locus (Fig. 1*b*), we also characterized new polymorphisms to assess the expression levels of the PGK and 129 alleles of the *Rps4* gene and also the *Smcx* gene, which escapes X inactivation in adult mouse tissues[32,33]. A polymorphism in the *Zfx* gene was used to quantify genomic DNA.

A summary of results from the SNuPE analysis is shown in Fig. 4*a*. Analysis of all three genes in PGK12.1 and XT67E1 undifferentiated ES cells shows that both alleles are expressed equally, as would be expected for cells with two active X chromosomes. In tissues from control ($129 \times$ PGK) F_1 females and differentiated parent PGK12.1 cells (EB), a significantly higher level of PGK strain RNA was observed, consistent with the

expected *Xce* effect. In marked contrast, a significantly lower level of transcripts from the PGK strain allele was observed for all three genes in differentiated targeted XT67E1 cells. Quantitative slot-blot analysis of *Oct4* RNA (Fig. 4*b*) indicates extensive, and equivalent, differentiation of PGK12.1 and XT67E1 cultures ($13.7 \pm 3\%$, $n = 4$, and $16.8 \pm 3.8\%$, $n = 5$, of mean ES cell value, respectively). The *Zfx* DNA controls indicate that the skewed allelic RNA levels in XT67E1 cells are not due to specific elimination of the PGK X chromosome. In adult tissues the *Xce* effect is considerably reduced for the *Smcx* gene, consistent with the fact that this gene escapes X inactivation, whereas in differentiated PGK12.1 cells the full *Xce* effect is apparent. This suggests that the *Smcx* allele on the inactive X chromosome undergoes near-complete X inactivation on differentiation of ES cells. This is supported by recent observations demonstrating that the *Smcx* gene only partly escapes X inactivation immediately after the onset of X inactivation *in vivo* (S.A.S., manuscript in preparation).

The allelic ratios for X-linked RNAs in XT67E1 cells is therefore skewed in the opposite direction to the *Xce* effect expected and observed in control PGK12.1 cells. To determine the basis for this, we performed SNuPE analysis of *Pgk-1* RNA following reverse transcription–polymerase chain reaction (RT–PCR) on individual cells (examples are shown in Fig. 4*b*). Both alleles of *Pgk-1* were detected, frequently at nearly equal levels, in all undifferentiated PGK12.1 and XT67E1 ES cells. Monoallelic expression, either of the PGK or the 129 strain allele, was observed in control cells from an adult female $(PGK \times 129)F_1$ mouse (Fig. 4*b*, cells 7–12), and also from differentiated PGK12.1 cells (Fig. 4*b*, cells 15, 16 and 18), consistent with random X inactivation. In marked contrast, XT67E1 cells showed monoallelic expression of the 129 allele only (Fig. 4*b*, cells 19, 21, 22, and 24), indicating complete non-random X inactivation of the PGK derived X chromosome. A summary of the single-cell analysis is shown in Fig. 4*c*. SNuPE assays were quantified, and cells showing > 80% levels of one allele were scored as having undergone X inactivation (see Fig. 4 Methods). Cells with nearly equal levels of both alleles ($50 \pm 10\%$) were scored as having two active X chromosomes. This analysis clearly demonstrates that differentiated PGK12.1 and control bone-marrow cells undergo random X inactivation, whereas XT67E1 cells undergo complete non-random X inactivation of the X chromosome bearing the non-targeted *Xist* allele. The proportion of cells with no inactive X chromosome was higher in the differentiated XT67E1 than the PGK12.1 cultures (see Discussion).

X inactivation *in vivo*

To analyse further the phenotype of XT67E1 cells, we assessed X inactivation *in vivo* by making aggregation chimaeras of parent PGK12.1 or targeted XT67E1 cells with eight-cell CD1 host embryos. RNA isolated from chimaeric embryos at 10.5–12.5 days post coitum (d.p.c.) was analysed using the SNuPE assay. Examples of the results obtained are shown in Fig. 5. Parent PGK12.1 cells exhibit random X inactivation in chimaeric embryos, as indicated by the expression of both alleles of *Xist* and *Pgk-1* in a PGK12.1 XY host chimaeric embryo. The high relative level of the 129 allele of *Pgk-1* and *Smcx* is due to the host embryo contribution (the CD1 and 129 X chromosome carry the same alleles). Random X inactivation of PGK12.1 cells, as indicated by expression of the PGK allele of *Xist* and *Pgk-1*, was also observed in two XX host chimaeric embryos (not shown). In contrast to this, examination of targeted XT67E1 cells demonstrates complete non-random X inactivation of the PGK strain X chromosome bearing the intact *Xist* locus. Thus, in XY host chimaeric embryos, only the PGK strain *Xist* allele is detectable owing to the absence of host embryo expression. In XX host chimaeric embryos, expression of the CD1 strain *Xist* allele from the host embryo is seen, as

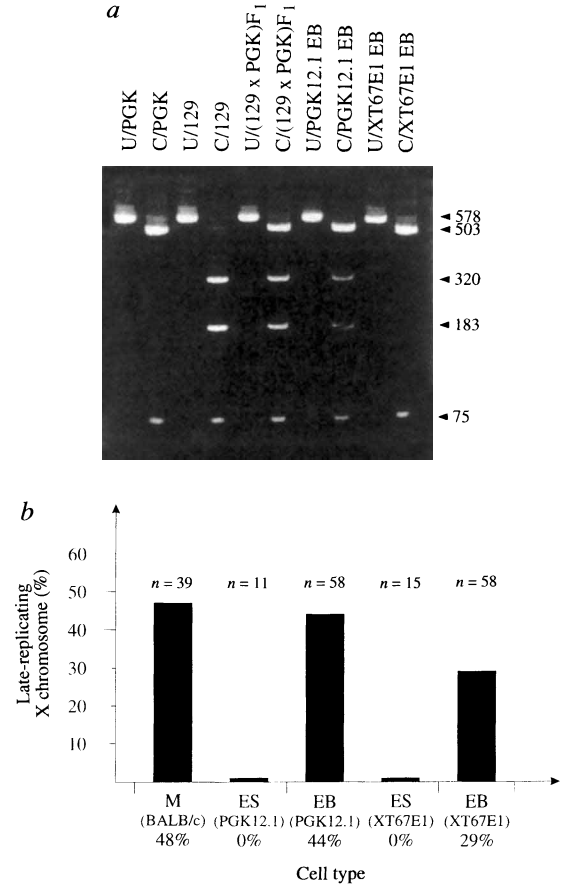

FIG. 3 XT67E1 cells undergo initiation of X inactivation on differentiation *in vitro*. *a*, PGK12.1 and XT67E1 cells were analysed for *Xist* expression following differentiation. A *Hin*dIII polymorphism was used to distinguish 129 and PGK strain *Xist* alleles[20] (U, uncut; C, cut with *Hin*dIII). Band sizes are in base pairs. Controls were adult female tissue RNA from PGK and 129 strain, and a $(129 \times PGK)F_1$ mouse. Differentiated (EB) PGK12.1 cells expressed both *Xist* alleles, and XT67E1 cells only the PGK strain allele, consistent with deletion of minimal promoter sequences from the 129 allele. *b*, Presence of a late-replicating inactive X chromosome in differentiated XT67E1 cells was demonstrated by a cytogenetic assay[29]. Positive controls were differentiated PGK12.1 cells, and primary cultured thymocytes from a female BALB/c mouse. Negative controls were provided by undifferentiated PGK12.1 and XT67E1 ES cells. A double-blind experiment, scoring metaphase spreads with 40 chromosome karyotype for the presence or absence of a pale-staining (late-replicating) X chromosome was performed. No late-replicating X chromosome was detected in spreads from XT67E1 and PGK12.1 ES cells (both X chromosomes active), but in their differentiated derivatives (EB), and also control primary thymocytes from female BALB/c mice (M), late-replicating inactive X chromosomes were observed. The frequency of late-replicating chromosomes in differentiated PGK12.1 controls was consistent with that determined in previous studies[28,30], but was reduced in differentiated XT67E1 cells.
METHODS. ES cells were differentiated as described in Fig. 1. The allele-specific *Xist* RT–PCR assay was performed as described previously[18]. The late-replication assay was a modification of that described previously[24]. Cells were cultured with $200\,\mu g\,ml^{-1}$ BrdU for 8 h and then $50\,ng\,ml^{-1}$ colchicine for 1 h. BALB/c thymocytes were cultured in EB medium with $4\,\mu g\,ml^{-1}$ concanavalin A for 3 days before addition of BrdU. Metaphase spreads were prepared by dropping acetic acid/methanol-fixed cells onto microscope slides. After ageing for several days, slides were stained with Acridine Orange in Gurrs buffer, pH 6.8, mounted and viewed under fluorescence on a Zeiss Axiophot. Slides were coded and then independently scored by two people.

well as the PGK strain *Xist* allele from donor cells. Analysis of the *Pgk-1* gene, however, demonstrates complete absence of expression of the PGK strain allele in XY and XX host chimaeric embryos, demonstrating that donor cells have all inactivated the PGK strain-derived X chromosome. The *Smcx* gene, which partly escapes X inactivation, is expressed from both alleles. The higher relative level of 129 alleles of *Pgk-1* and *Smcx* is again due to host embryo contribution. Identical results were obtained in three additional chimaeric embryos (not shown). The *in vivo* analysis therefore confirms the *in vitro* findings, and clearly demonstrates that XT67E1 cells do not inactivate the 129 X chromosome bearing the deleted *Xist* allele.

Discussion

We have generated an XX ES cell line, PGK12.1, which carries genetically distinct PGK and 129 strain X chromosomes. We analysed clonal derivatives of this cell line and showed that both alleles of *Xist* and the *Pgk-1* gene are expressed at levels consistent

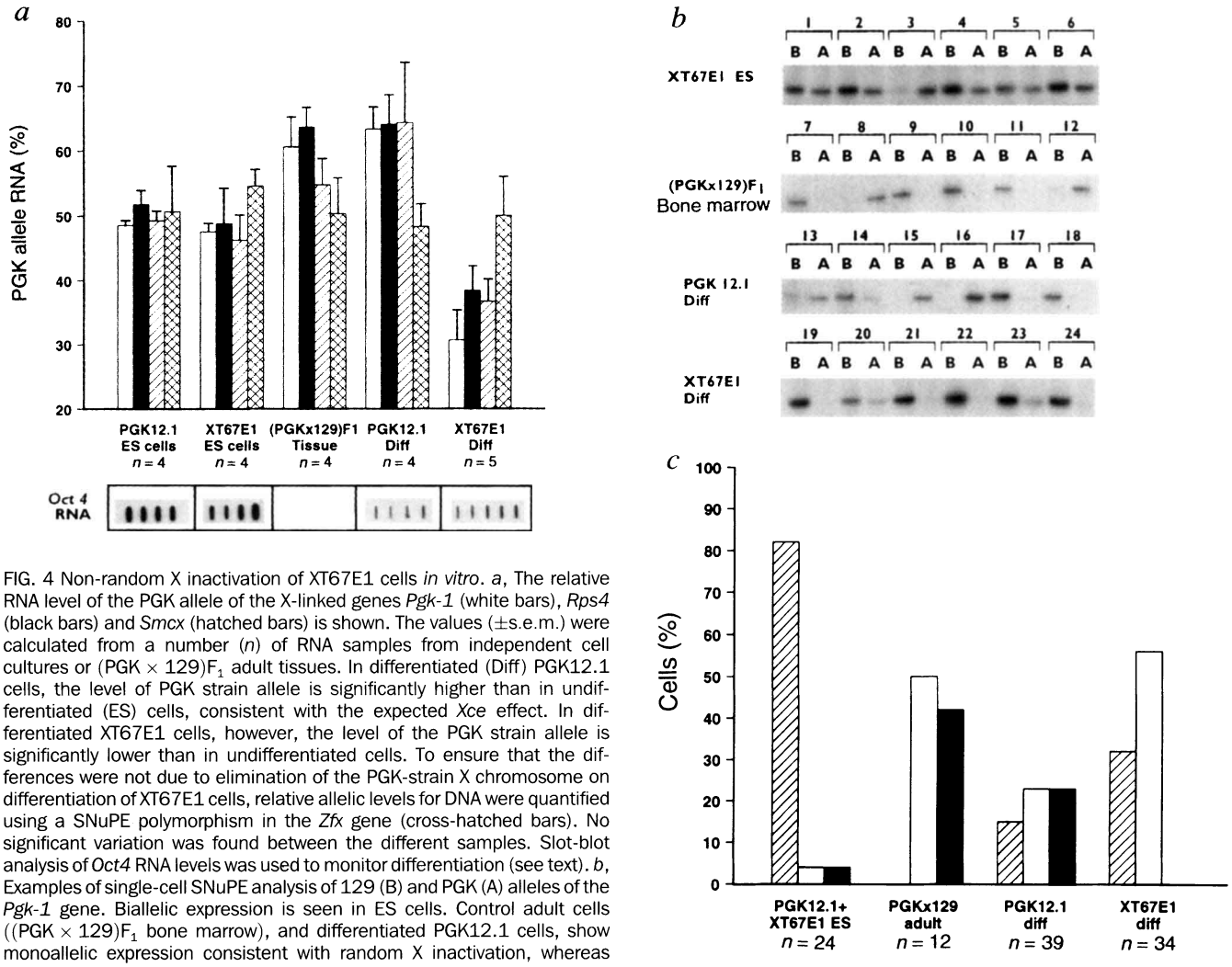

FIG. 4 Non-random X inactivation of XT67E1 cells *in vitro*. *a*, The relative RNA level of the PGK allele of the X-linked genes *Pgk-1* (white bars), *Rps4* (black bars) and *Smcx* (hatched bars) is shown. The values (±s.e.m.) were calculated from a number (*n*) of RNA samples from independent cell cultures or (PGK × 129)F_1 adult tissues. In differentiated (Diff) PGK12.1 cells, the level of PGK strain allele is significantly higher than in undifferentiated (ES) cells, consistent with the expected *Xce* effect. In differentiated XT67E1 cells, however, the level of the PGK strain allele is significantly lower than in undifferentiated cells. To ensure that the differences were not due to elimination of the PGK-strain X chromosome on differentiation of XT67E1 cells, relative allelic levels for DNA were quantified using a SNuPE polymorphism in the *Zfx* gene (cross-hatched bars). No significant variation was found between the different samples. Slot-blot analysis of *Oct4* RNA levels was used to monitor differentiation (see text). *b*, Examples of single-cell SNuPE analysis of 129 (B) and PGK (A) alleles of the *Pgk-1* gene. Biallelic expression is seen in ES cells. Control adult cells ((PGK × 129)F_1 bone marrow), and differentiated PGK12.1 cells, show monoallelic expression consistent with random X inactivation, whereas differentiated XT67E1 cells show monoallelic expression of the 129 allele only. *c*, Summary of quantified single-cell analysis. Cells showing 50 ± 10% levels of both alleles were scored as having no inactive X chromosome (hatched bars). Cells with > 80% 129 (white bars) or PGK (black bars) allele were scored as having undergone X inactivation (see Methods).

METHODS. Cell culture, *Oct4* analysis and SNuPE analysis were as described in Fig. 1. New polymorphisms were found for the *Rps4* gene[41] (position 399; PGK strain, G; 129 strain, A), the *Smcx* gene[32] (position 228 of the published partial cDNA sequence; PGK strain, C, 129 strain, G) and the *Zfx* gene[42] (position 4311; PGK strain, T; 129 strain, A). Primers for *Rps4*; Rps4-323; AGGGTCGCTTTGCTGTTCA and Rps4-885; AGTTTCACCATGCTGTTTA (*Rps4* RT–PCR, 30 cycles of 94 °C 1′, 52 °C 1′, 72 °C 1′) and Rps4-358; CAGGTGCGGGATTCCTTTTGTGCC (*Rps4* SNuPE, 1 cycle of 94 °C 1′, 62 °C 2′, 72 °C 1′). For *Smcx*; Smcx-55; TGGTTCCTTGCTACGCTCTCACTA and Smcx-889; CAGCCGCCAAAACTCCTTCTCTAC (*Smcx* RT–PCR, 30 cycles of 94 °C 1′, 55 °C 1′, 70 °C 1′), and Smcx-204; CTGTGCAGCCTTCCAAGTTCAACA (*Smcx* SNuPE, 1 cycle of 94 °C 1′, 59 °C 2′, 72 °C 1′), and for *Zfx*; Zfx-4068; GTGAACGGGTGCTAAGGAGGACT, and Zfx-4458; TAACGGCACGAAAGGGATGG (*Zfx* PCR, 30 cycles of 94 °C 1′, 55 °C 1′, 72 °C 1′), and Zfx-4312; AAAGACATCTTAACAAAAATCTTAATA (*Zfx* SNuPE, 1 cycle of 94 °C 1′, 45 °C 2′, 72 °C 1′). Single-cell analysis was performed by diluting trypsinized single-cell suspensions at 10^4 cells per ml and pipetting 0.2 μl microdrops into 96-well plates. Lysis solution (10 μl; 0.05% NP40, 1 mg ml^{-1} glycogen, 10 μl ml^{-1} RNasin) was added to wells containing a single cell. Negative PCR controls were provided by microdrops without cells. Samples were ethanol precipitated and redissolved in 5 μl of RT mix. After RT (using gene-specific primer, Pgk750 (ref. 24)), PCR mix (65 μl) was added directly to samples, and PCR (40 cycles) was performed with primers Pgk750 and Pgk402 (CCTCCGCTTTCATGTAGAGGAAGA). SNuPE was performed as described previously[24]. Control bone-marrow cells were cultured as described previously[43]. Several differentiated PGK12.1 and XT67E1 single cells showed near-complete monoallelic expression, rather than complete monoallelic expression (see, for example, Fig. 4*b*, cells 13, 14, 17 and 23), presumably because they are contaminated by trace amounts of cell debris and RNA owing to high levels of apoptosis in differentiated cultures, and/or cell damage arising from the preparation of single-cell suspensions. Also, differentiated cultures will contain cells that have recently undergone X inactivation, and therefore have trace amounts of RNA from the inactive allele still present. We therefore scored cells with > 80% monoallelic expression as having undergone X inactivation.

with random X inactivation subject to the predicted *Xce* effect. Gene targeting by homologous recombination was used to create a 7-kb deletion of the 129 derived *Xist* gene resulting in a null allele. On differentiation *in vitro*, targeted cells undergo X inactivation, indicating that the counting function of the Xic is unaffected. However, unlike parent PGK12.1 cells, targeted cells differentiated *in vitro* or *in vivo* display complete non-random X inactivation of the PGK strain X chromosome bearing the intact *Xist* allele. These results show that *Xist* is required *in cis* for X inactivation to occur.

Non-random X inactivation in XT67E1 cells may be occurring as a result of either primary or secondary mechanisms, as illustrated in the models in Fig. 6. Primary non-random X inactivation would result if the *Xist* deletion disrupts the choice function of the Xic, that is, the XT67E1 cells always inactivate the PGK X chromosome bearing the non-targeted *Xist* locus (Fig. 6*a*). The

Allele	PGK 129/CD1	PGK 129/CD1	PGK 129/CD1	PGK 129/CD1
Xist				
Pgk - 1				
Smcx				
Host embryo	XY	XX	XX	XY
Cell line	XT67E1			PGK12.1

FIG. 5 XT67E1 cells show complete non-random X inactivation *in vivo*. Chimaeric embryos were made by aggregation of eight-cell CD1 host embryos with either XT67E1 or PGK12.1 donor ES cells. RNA was prepared from embryos 10.5–12.5 d.p.c.. SNuPE analysis was performed using polymorphisms in the *Xist*, *Pgk-1* and *Smcx* genes. The presence of a Y chromosome indicates the chromosomal sex of the host embryo. This was determined by PCR for the *Zfy* gene from yolk sac DNA as described previously[18]. In an XY host embryo, the PGK12.1 cells expressed both the PGK and 129 strain *Xist* alleles, and the PGK and 129/CDI strain *Pgk-1* alleles, consistent with random X inactivation. Targeted XT67E1 cells, however, expressed the PGK *Xist* allele and only the 129/CD1 *Pgk-1* allele in XX or XY host embryos, indicating that all donor cells have an inactive PGK-derived X chromosome. Both alleles of the *Smcx* gene were expressed in all chimaeric embryos, consistent with this gene escaping X inactivation.

METHODS. Aggregation chimaeras were prepared with eight-cell CD1 host embryos, essentially as described previously[44], except that ES cell clumps were produced by a short trypsinization, stopped by addition of M16 with 10% fetal calf serum, and aggregations were performed by laying small (4–12 cell) ES cell clumps onto compacting eight-cell embryos placed in microwells. After overnight incubation in M16 (5% CO_2, 37 °C, 100% humidity), aggregates were transferred in M16 with 10% fetal calf serum to the uterine horns of day 2.5 pseudopregnant CD1 foster mothers. Chimaeric embryos were collected from recipient mothers at 10.5–12.5 d.p.c. SNuPE analysis of RNA from chimaeric embryos was as described in Figs 1 and 4.

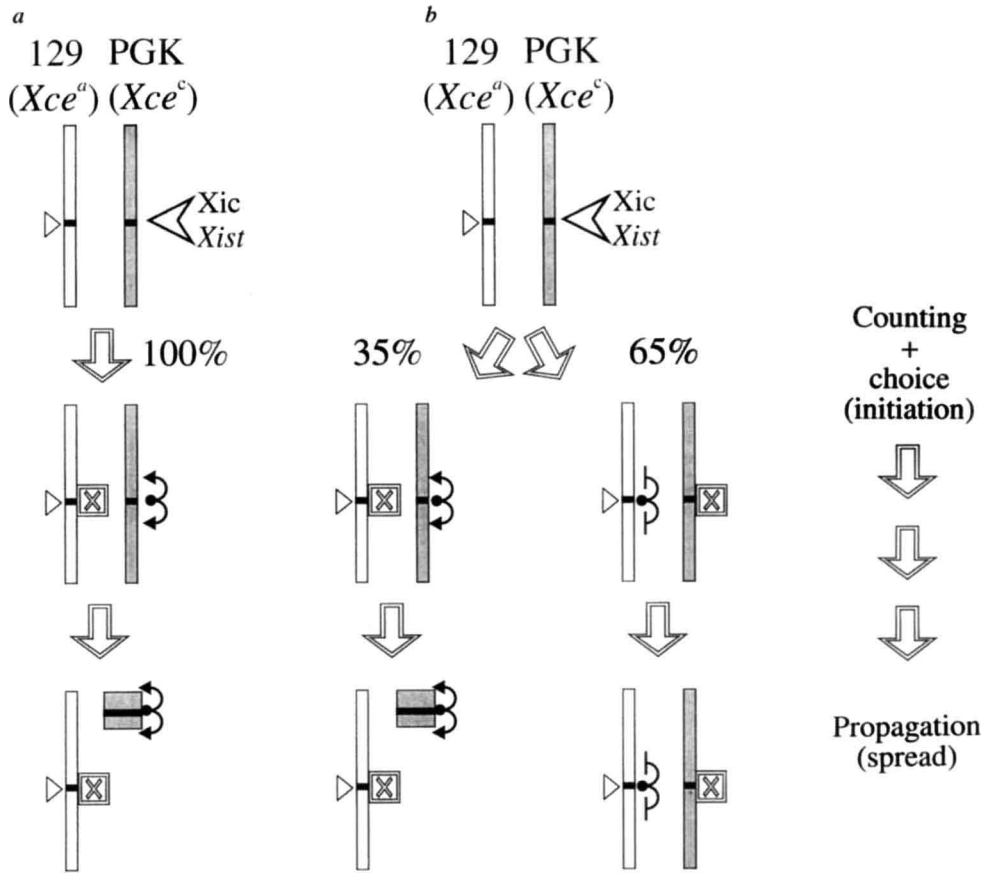

FIG. 6 Primary and secondary non-random X inactivation models for targeted XT67E1 cells. These models are based on the proposal that each cell has a limited amount of a blocking factor (indicated by open squares with a cross), sufficient to bind and repress a single Xic[4]. *In cis* propagation of X inactivation is triggered from unblocked Xics on differentiation of cells from the totipotent embryonic lineage. This proposal is consistent with the observation that one X chromosome remains active per diploid autosome set in sex-chromosome aneuploid[45] and tetraploid cells[46]. *a*, Primary non-random X inactivation results if the deleted *Xist* allele on the 129 X chromosome (white bar) is blocked in all cells. *Xist* expression (curved arrows) proceeds on the PGK X chromosome (grey bar), resulting in an inactive PGK X chromosome (grey square) in all cells. *b*, Secondary non-random X inactivation results if either *Xist* allele can be blocked, but cells in which the normal allele is blocked fail to express *Xist* and do not propagate X inactivation *in cis* from the deleted allele (blunt curves). Cells which block the deleted *Xist* allele, however, express *Xist* from the PGK X chromosome and go on to X inactivate *in cis*. The proportion of these two cell populations is governed by the *Xce* effect as indicated, but differentiated cells with two active X chromosomes will be selected against as a result of failure to dosage compensate the X chromosome.

Xist deletion would then be equivalent to a very strong *Xce* allele. Secondary non-random X inactivation would result if the counting and choice functions of the Xic are unaffected by the *Xist* deletion, and cells that elect to inactivate the 129 X chromosome (bearing the targeted *Xist* locus) fail to X inactivate in *cis*. Cells that elect to inactivate the PGK X chromosome (bearing an intact *Xist* locus), however, will undergo X inactivation (Fig. 6*b*).

Although we cannot state definitively which mechanism is operating in XT67E1 cells, our data favour secondary non-random X inactivation. Analysis of *Oct4* expression indicates *in vitro* differentiation levels greater than 80%, both for PGK12.1 and XT67E1 cells (Fig. 4*a*). On this basis, primary non-random X inactivation (Fig. 6*a*) would lead to predominant expression of the 129 allele of X-linked genes in differentiated cells. We observed 129 allele values of 65%, 63% and 67% for the *Pgk-1*, *Rps4* and *Smcx* genes, respectively (Fig. 4*a*). These values are more consistent with the secondary non-random X inactivation model (Fig. 6*b*); according to this model the proportion of cells inactivating the PGK- and 129-derived X chromosomes will be governed by the *Xce* effect, as in control cells. Thus, in differentiated XT67E1 cells, approximately 65% of cells would inactivate from the targeted 129 *Xist* allele, and therefore go on to differentiate into cells with both X chromosomes active. The predicted level of 129 strain RNA for an X-linked gene undergoing X inactivation would therefore be 60.6%, compared to the 35% level predicted and observed in control PGK12.1 cells. That we see slightly higher levels of the 129 allele than predicted by this model could reflect selection *in vitro* against differentiated cells with two active X chromosomes. The single-cell analysis (Fig. 4*c*) and the late-replication analysis (Fig. 3*b*) indicate that the proportion of XX active cells is higher in differentiated XT67E1 cultures than in differentiated PGK12.1 cultures, consistent with the secondary non-random X inactivation model. In both cases this difference is less than predicted in the model, but again this could be accounted for by selection *in vitro* against differentiated cells with two active X chromosomes. The complete non-random X inactivation observed *in vivo* is consistent either with primary or secondary non-random X inactivation, as T(X;16)16H/+ cells which are functionally disomic for the proximal half of the X chromosome are rapidly selected against after the onset of X inactivation[34,35].

It is possible that the non-random X inactivation observed results from an unrelated, spontaneous cell-lethal mutation on the 129 X chromosome, but such a mutation would need to result in extremely rapid cell death *in vitro*, as no single cells with an inactive PGK X chromosome were seen (Fig. 4*c*). We therefore consider this possibility highly unlikely.

In summary, our data demonstrate that an intact *Xist* gene is required *in cis* for X inactivation to occur. We propose that the phenotype of XT67E1 cells results from secondary non-random X inactivation of the chromosome bearing the non-deleted *Xist* allele, and that the counting and choice functions of the Xic are not affected by the deletion. If correct, this would support our previous suggestion that the primary decision determining how many and which X chromosomes to inactivate involves the choice of which, if any, *Xist* alleles are expressed, and that subsequent propagation of X inactivation in *cis* results from expression of the *Xist* gene[16,18]. We predict that the sequences required for counting and choice reside in regulatory elements of the *Xist* gene. These elements may lie in the region 5′ of the gene, but could also be located 3′ to *Xist*, consistent with genetic mapping studies which suggest that *Xce* lies distal to *Xist*[36]. Further studies will be required to determine the mechanism of *Xist* function, as our data are consistent both with models that propose *Xist* RNA as the functional *cis*-inactivating signal[16,17] and those that propose the underlying chromatin structure of the active *Xist* locus as the primary determinant of *cis* inactivation[16]. □

Received 18 August; accepted 24 November 1995.

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ACKNOWLEDGEMENTS. We thank N. Takagi for advice on late-replication assays; A. Fisher for help with bone-marrow tissue culture; and R. Beddington, V. Episkopou and members of the Section of Comparative Biology for valuable discussions. This work was supported by the Medical Research Council of Great Britain.