

# Asynchronous replication and allelic exclusion in the immune system

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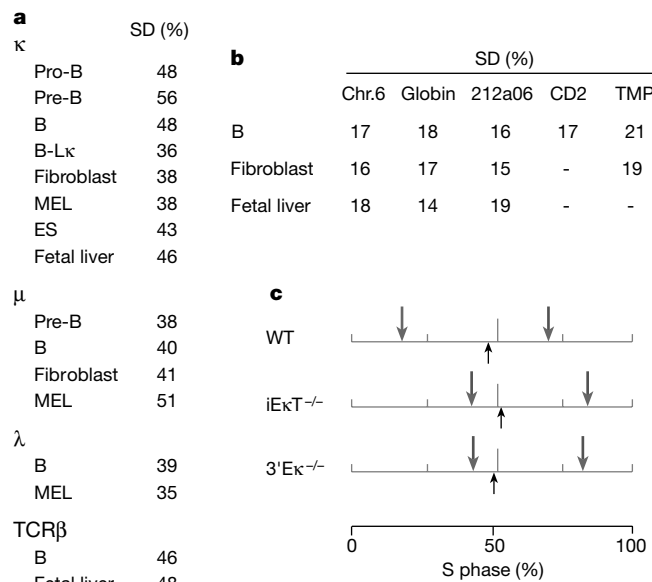
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The development of mature B cells involves a series of molecular decisions which culminate in the expression of a single light-chain and heavy-chain antigen receptor on the cell surface<sup>1,2</sup>. There are two alleles for each receptor locus, so the ultimate choice of one receptor type must involve a process of allelic exclusion. One way to do this is with a feedback mechanism that downregulates rearrangement after the generation of a productive receptor molecule<sup>3</sup>, but recent work suggests that monoallelic epigenetic changes may also take place even before rearrangement<sup>4</sup>. To better

understand the basis for distinguishing between alleles, we have analysed DNA replication timing. Here we show that all of the B-cell-receptor loci ( $\mu$ ,  $\kappa$  and  $\lambda$ ) and the TCR $\beta$  locus replicate asynchronously. This pattern, which is established randomly in each cell early in development and maintained by cloning, represents an epigenetic mark for allelic exclusion, because it is almost always the early-replicating allele which is initially selected to undergo rearrangement in B cells. These results indicate that allelic exclusion in the immune system may be very similar to the process of X chromosome inactivation.

The entire genome is divided into a series of well defined zones which replicate in a programmed manner throughout the S phase of the cell cycle, and this pattern is correlated with gene expression. Housekeeping genes all replicate in early S phase, whereas many tissue-specific genes are developmentally regulated to replicate late in most tissues, but early in the cells that express these genes<sup>5</sup>. Replication timing is easily assayed by fluorescence *in situ* hybridization (FISH) analysis of interphase nuclei, which reveals a single dot for an as-yet-unreplicated locus and a double signal after replication<sup>6</sup>. In a non-synchronized population of cells, a high percentage of double signals is indicative of early replication, and a preponderance of single dots is characteristic of late-replicating DNA. Monoallelically expressed sequences, such as imprinted genes<sup>7</sup>, olfactory receptors<sup>8</sup> or the X chromosome in females<sup>9</sup>, all show an asynchronous replication pattern whereby one allele replicates earlier than the other in each cell. This is easily detected by the presence of a high percentage of cells showing one single and one double hybridization signal in the FISH assay<sup>7</sup>.

Because antigen receptor genes are expressed monoallelically in the lymphoid lineage, we examined these loci for replication timing patterns using FISH. Initial studies were carried out for the  $\kappa$  light-chain gene in normal B cells. Strikingly, over 40% of interphase



**Figure 1** Antigen receptor loci replicate asynchronously. **a**, Cell lines or stimulated B and T cells were labelled with BrdU and then prepared and assayed for replication timing using probes for the antigen receptor loci. For each experiment the number of cells with single/single (SS), double/double (DD) or single/double (SD) signals were recorded, but only the %SD is shown in the chart. In each case 100–300 nuclei were counted. In another experiment (not shown), probes for C $\kappa$  alone (35% SD), C $\kappa$  together with the most 3' V $\kappa$  sequences (36% SD), V $\kappa$  located 2 Mb upstream (36% SD) and R $\beta$ i located 40 kb downstream (35% SD) all showed similar asynchronous replication, indicating that this pattern is regional, and the same was true for C $\mu$  (35% SD) and a probe located about 170 kb downstream of C $\mu$  (37% SD) at the heavy-chain locus. B-L $\kappa$  are B cells from a mouse carrying a transgenic  $\kappa$  locus<sup>15</sup>. The unrearranged endogenous alleles were

assayed using the R $\beta$ i probe, which represents a region not included in the transgene. **b**, The %SD for several control probes is shown. Additional controls show synchronous replication (<20%SD) for erythroleukaemia (MEL) and F9 (which is similar to embryonic stem cells, ES); see ref. 7. **c**, Replication timing was measured at the  $\kappa$  locus (using C $\kappa$ ) in B cells from a wild-type (WT) mouse and from mice carrying homozygous targeted deletions for the intronic (iE $\kappa$ T<sup>-/-</sup>) or the 3' (3'E $\kappa$ T<sup>-/-</sup>)  $\kappa$  enhancers. The results are shown graphically, with the first arrow (from the left) representing the percentage of SS cells and the second arrow representing the percentage of SS + SD cells. This presentation allows us to visualize (upper, thick arrows) the time in S phase when the first and second alleles replicate. The average percentage of single signals for a control probe (CD2) is shown (lower, thin arrows) for each cell type.

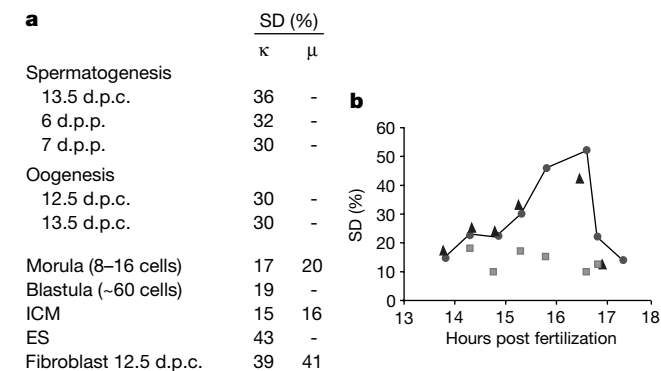
nuclei show a single/double pattern (Fig. 1a), indicating that the two alleles at this locus indeed replicate asynchronously, in contrast to a number of different control genes which show a low level of single/double signals (Fig. 1b). A similar pattern was observed in proliferating cells from earlier stages of B-cell development and even in non-lymphoid cells types, such as erythroleukaemia or primary fibroblasts, suggesting that the pattern must be set up early in development (Fig. 1a). Indeed, unlike other structural features of the  $\kappa$  locus which are specific for B cells<sup>10</sup>, the control of this phenomenon is not affected by the major  $\kappa$  enhancers, and when they are removed by targeted deletion<sup>11,12</sup>, overall replication timing shifts to a later time, but the two alleles still undergo DNA synthesis asynchronously (Fig. 1c). We note that in addition to the  $C_\kappa$  probe used in these experiments, probes for the  $V_\kappa$  region (BAC 122K2) located 2 Mb upstream and the ribose 5-phosphate isomerase (*Rpi*) gene located 40 kb downstream<sup>13</sup> also showed asynchronous replication (35–36% single/double) (details in legend to Fig. 1), and similar results were obtained for the  $\mu$  locus (see legend to Fig. 1), indicating that this is a regional phenomenon with a pattern very similar to that observed for imprinted gene domains<sup>7</sup>. Furthermore, this asynchronous replication profile is not unique to the  $\kappa$  locus, and similar data were obtained for the B-cell-specific  $\lambda$  light chain and  $\mu$  heavy chain<sup>31</sup>, as well as the T-cell-associated TCR- $\beta$  locus (Fig. 1a).

Asynchronous replication of antigen receptor loci is not restricted to the lymphoid system and can be observed in a variety of different cell types, so we next asked when this pattern is set up during development. Analysis of replication timing in sequential stages of spermatogenesis and oogenesis demonstrated that the two  $\kappa$  alleles replicate asynchronously before meiosis (Fig. 2a). This differential marking is evidently retained in mature germ cells as well, since the same pattern was observed again immediately after fertilization during the first division cycle in zygotes (Fig. 2b). This pattern is clearly lost, however, in the morula and blastula, where both alleles

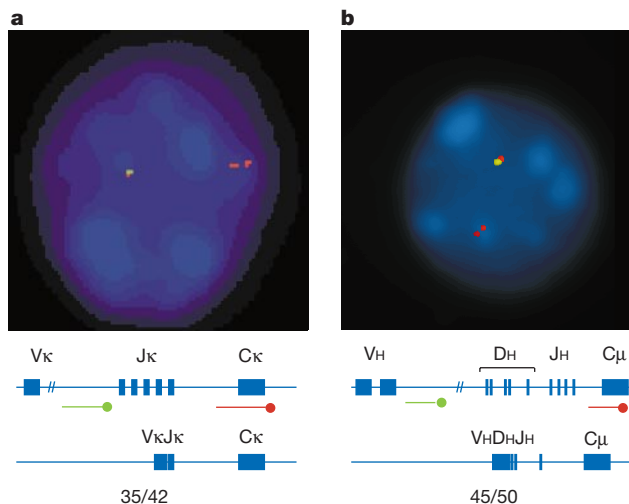
replicate relatively synchronously (about 20% single/double), and this erasure was even observed in purified inner cell mass (ICM) cells, which actually serve as the source for the embryo proper. Asynchronous replication was again observed in embryonic stem cells (which mimic the post-blastula embryo), and in early fibroblasts. A similar phenomenon was observed for the  $\mu$  heavy-chain locus, as well (Fig. 2a, b). This pattern is distinctly different from those of imprinted genes: such genes are programmed in the gametes to replicate in a way that is specific to the parent of origin; they then maintain this asynchronous pattern through pre-implantation development<sup>14</sup> (see legend to Fig. 2a). In contrast, differential replication timing at the antigen receptor loci is erased in the morula and then re-established, probably at about the time of implantation. The monoallelically expressed olfactory receptor genes and IL-4 behave in a similar manner<sup>14</sup>.

The establishment of the observed asynchrony of replication well before B-cell development suggests that it could provide a differential mark, rendering one allele more accessible for rearrangement. With this in mind, we wanted to test whether asynchronous replication timing is itself correlated with the selection of a single allele for rearrangement. Thus we devised an *in situ* system capable of distinguishing between the germline and rearranged copies of the  $\kappa$  locus. This was done by using two separate FISH probes, one for  $C_\kappa$ , and a second which hybridizes upstream of the  $J_\kappa$  segments in a region normally deleted as part of the rearrangement process. An analysis of over 40 B-cell nuclei exhibiting an asynchronous replication pattern (single/double) for the  $C_\kappa$  probe shows that in 83% of the cases, the late-replicating copy (single dot) is associated with the germline (non-deleted) allele (Fig. 3a). These findings indicate that rearrangement in B cells almost always occurs on the early-replicating allele, and this appears to be a general phenomenon for the antigen receptor loci, since analysis of the  $\mu$  locus gave almost identical results (90% of cases) (Fig. 3b).

The asynchronous replication timing pattern that we observe appears to be independent of antigen receptor rearrangement and



**Figure 2** Asynchronous replication during development. **a**, Replication timing analysis of the  $\kappa$  locus in cells for various stages of gametogenesis and in the early embryo, including purified ICM. As a control, the imprinted gene *Snrpn* was also tested and found to be asynchronously replicating in ICM cells (33% SD). In each case 100–200 nuclei were scored for SS, SD and DD signals but only the percentage SD is shown in the chart. Results using control probes for cells in gametogenesis and for pre-implantation embryos (morula and blastula) have been published<sup>14</sup> and shown to be statistically different from probes demonstrating asynchronous replication. **b**, A mixture of zygotes from 15–20 superovulated females was incubated and assayed for SD signals by FISH at the indicated time on the day after mating using  $\kappa$  (circles),  $\mu$  (triangles) and control (squares) probes. Since all of the zygotes undergo their first round of replication at about the same time, we see a peak for the SD pattern (16–17 h post fertilization). Premeiotic germ cells presumably have one early and one late allele in each cell. Thus, zygotes could be formed with either two early, two late or one early and one late allele, implying that only 50% of the cells should show asynchrony at the peak time. We note that for imprinted genes, where all of the zygotes carry asynchronous alleles, the maximum level of SD signals was much higher<sup>14</sup>. d.p.c., days post coitum; d.p.p. days post partum.



**Figure 3** Rearrangement is associated with early replication. **a**, B cells were assayed by FISH using a probe for  $C_\kappa$  (pSP1g8) (red) and a second probe for the region upstream to  $J_\kappa 1$  (pGL1.6) (green) which is deleted in most rearranged cells. Nuclei showing a single/double signal for  $C_\kappa$  were scored for the position of the intact germline allele in the same cell. In 35 of 42 cells, the germ line allele was associated with the single signal. It should be noted that in the mouse, rearrangement can also occur by inversion, in which case the upstream  $J_\kappa 1$  probe is not deleted, but rather shifted to a distal position. Cells of this nature were not included in the survey. **b**, A similar experiment was done using a  $C_\mu$  probe (red) and a second probe upstream to the D region (green). In 45 of 50 cells, the allele which did not undergo V to DJ rearrangement is associated with the single, unrearranged signal.

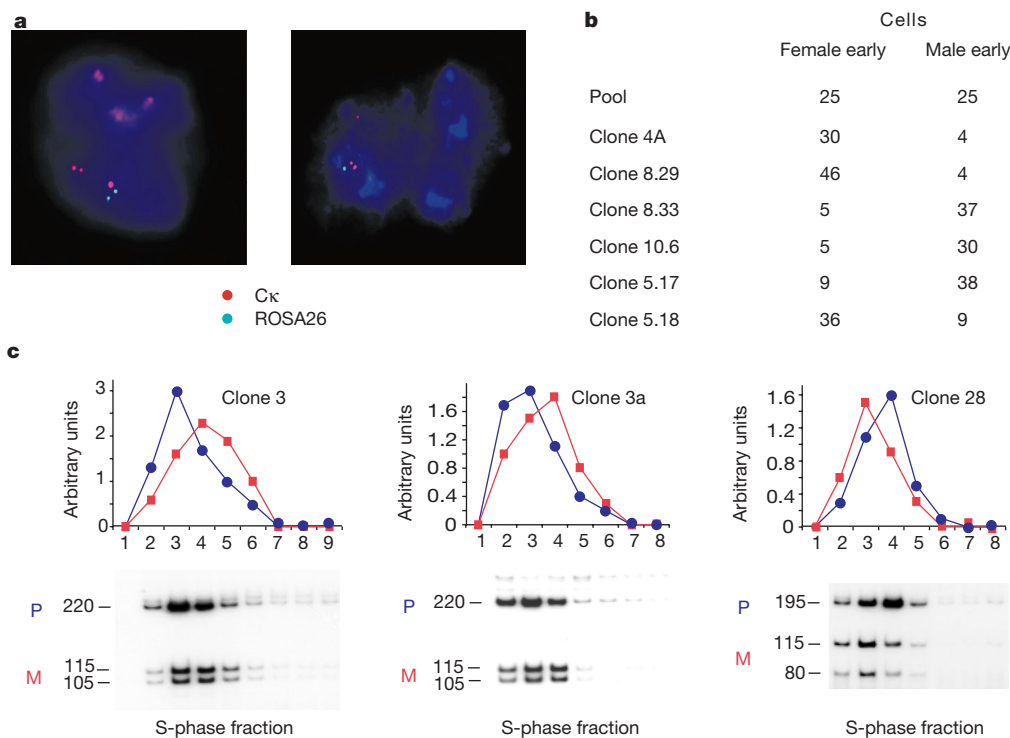
expression. In spleen cells carrying a  $\kappa$  transgene (B-L $\kappa$ ), for example, both endogenous  $\kappa$  loci are in the silent germline configuration and not expressed<sup>15</sup>, yet these alleles still replicate asynchronously (36% single/double) (see Fig. 1a). We also analysed replication timing in B cells from knock-in mice carrying a pre-rearranged  $\kappa$  gene on both alleles. These B cells express both alleles at a high level (R.M., Y.B, H.C. & K. Rajewsky, unpublished results), yet still have asynchronous replication of the  $\kappa$  alleles (36% single/double).

Because the initial rearrangement at the  $\kappa$  locus can occur randomly on either the maternal or paternal allele in each cell<sup>16</sup>, we assumed that early-replication timing must also be set up in a stochastic manner: some cells would have an early-replicating maternal allele and others an early-replicating paternal allele. To test this hypothesis, we generated primary fibroblasts from a mouse that was heterozygous for an insertion of *neo-lacZ* into the ROSA26 locus on chromosome 6 (ref. 17), not far (approximately 18 centimorgans) from the  $\kappa$  locus; we then used this marker to identify the parental alleles in each cell. By double-label FISH we scored cells containing a single/double pattern for C $\kappa$ . In 50% of these nuclei, the paternal allele replicated early, while in the remainder of the nuclei the maternal allele did so. We then asked whether these patterns, once established, are maintained in a clonal manner. Fibroblasts were transformed by infection with a retrovirus encoding SV-40 large T antigen and individual clones were then expanded. We analysed a number of these clones for  $\kappa$  replication timing using the *neo-lacZ* probe to identify the parental origin of the alleles. Each clone demonstrated a consistent pattern of allele-specific early replication. In clones 4A, 8.29 and 5.18, for example,

the ROSA26 transgene was almost always associated with the double signal in cells showing an asynchronous pattern, whereas in clones 8.33, 10.6 and 5.17, the pattern was reversed (Fig. 4a, b).

To confirm these results, we used the alternate approach of cell-cycle fractionation to measure replication timing. We generated individual pre-B-cell clones transformed by infection with Abelson virus from *Mus spretus*  $\times$  *M. musculus* F<sub>1</sub> mice. We labelled replicating DNA with bromodeoxyuridine (BrdU) and then used FACS (fluorescence-activated cell sorting) to separate the cells into seven to eight S-phase fractions. BrdU DNA was then isolated from each fraction and assayed for sequence content by quantitative polymerase chain reaction (PCR)<sup>18</sup>, taking advantage of a restriction site polymorphism in the  $\kappa$  region to identify the two parental alleles. As shown in Fig. 4c, each individual clone displays a clear asynchronous replication pattern, with one allele replicating before the other. Out of five clones analysed by this technique, three had the paternal *M. spretus* allele replicating early (for example, clone 3), and two clones had the maternal *M. musculus* allele first to replicate (for example, clone 28). These patterns remained stable for over 50 generations in culture and even after subcloning (for example, clone 3a).

These studies clarify the mechanism of allelic exclusion in the immune system. Unlike models based on the equal availability of both alleles to rearrangement<sup>3</sup>, our results suggest that asynchronous replication timing represents a fundamental epigenetic difference between the two alleles in each cell and that this serves as the basis for initially choosing one copy to undergo demethylation and subsequent rearrangement. It is probably this feature, in fact, which keeps the remaining germline allele in a closed con-



**Figure 4**  $\kappa$  asynchronous replication is heritable. **a**, Embryonic fibroblasts from mice carrying the *neo-lacZ* marked maternal ROSA26 transgene were assayed by FISH using the C $\kappa$  (red) and a *neo-lacZ* probe (green) for the ROSA26 transgene. C $\kappa$  single/doubles were then scored for the location of ROSA26. In 50% (25/50) of the cells, the single was associated with the ROSA26-carrying maternal chromosome 6 (left) and in 50% the single was associated with the paternal allele (right). **b**, Pooled fibroblasts and individual ear fibroblast subclones originating from four mice (numbered 4, 5, 8 and 10) were assayed as above for the number of paternal early or maternal early nuclei among cells showing an asynchronous replication pattern. **c**, Replication timing was analysed by S-phase

fractionation of BrdU-labelled Abelson-virus-transformed pre-B cells (see Methods) isolated from *M. spretus*  $\times$  *M. musculus* F<sub>1</sub> mice. The  $\kappa$  locus was amplified by PCR and *HhaI* then used to distinguish between the paternal (blue) *M. spretus* allele (uncut) and the maternal (red) *M. musculus* allele (cut). For clones 3 and 3a we used primers 170-1 and K2 which yield a 220-bp product, while clone 28 was assayed using primers 170-1 and K4 (195-bp product). We repeated each of these experiments at least three times. PCR analysis of a control region (mouse globin) from clone 3 showed a completely synchronous replication pattern (data not shown).

formation and thus provides enough time for a productive antigen receptor molecule to interact with the cell surface and ultimately bring about feedback inhibition of the rearrangement machinery. In the absence of a productive receptor, we assume that given enough time, the second, later-replicating allele can also undergo both demethylation and rearrangement<sup>4</sup>. According to this model, receptor loci continue to retain their differential epigenetic marks even after the cells have already decided on a single expressing allele. This may help explain why further alterations as a result of antigen-induced receptor editing<sup>19</sup> or somatic hypermutation<sup>20</sup> appear to be restricted to the already rearranged alleles. We have not investigated how the replication timing pattern affects rearrangement, but it is likely that this mechanism is indirect, probably involving chromatin structure. In this sense, it may well combine with many other components which control the cell specificity and timing of rearrangement through DNA accessibility<sup>10,32</sup>.

The differential marking of two alleles in the same cell, as occurs for the antigen receptor loci, is reminiscent of the process of X-chromosome inactivation. In both cases the regions involved replicate asynchronously in all somatic cells<sup>9</sup>. At the local level, the  $\kappa$  gene itself behaves in a manner analogous to X-chromosome-linked tissue-specific genes, which also appear to undergo monoallelic demethylation and transcriptional activation, but only in the cell type of expression<sup>21,22</sup>. Developmentally, these processes are also quite similar. Just as in the X chromosomes, differences between the alleles at the antigen receptor loci are erased in the morula<sup>23</sup>, and asynchronous replication is then re-established at about the time of implantation<sup>9</sup>. In both cases, the choice of which allele replicates early is random, with either the maternal or paternal copy being 'picked'; once established, replication asynchrony is clonally inherited through future cell divisions. Furthermore, it is always the early-replicating allele which is active. Thus, we have shown that this process of monoallelic inactivation is not unique to the X chromosome, but can also take place, albeit in a regional manner, on autosomes as well. Other loci, such as the olfactory receptor gene clusters<sup>8</sup> and the cytokines *Il-2* and *Il-4* (ref. 24) also replicate asynchronously in a variety of different cell types and are expressed monoallelically, suggesting that this may represent a common early embryonic mechanism for distinguishing between alleles.

**Note added in proof:** Another observation consistent with asynchronous replication of the  $\mu$  heavy chain in mature B cells (Fig. 1) has been recently reported<sup>31</sup>. □

## Methods

### Embryos

Superovulated C57Black  $\times$  BALB/C F<sub>1</sub> female mice were mated with males and zygotes were removed from the oviducts about 14–20 h after hormone injection. Zygotes were incubated at 37 °C in M16 medium (Sigma) to obtain pre-implantation embryos at different stages of development, and ICM was purified by immunosurgery<sup>25</sup>.

### Cells

Fetal livers were dissected out of 12.5 d.p.c. (days post coitum) embryos and mechanically disrupted in a solution of RPMI + 10% FCS by passing through a syringe. The remainder of the embryo was disrupted with a 2-ml syringe into a solution of DMEM + 10% FCS in order to obtain embryonic fibroblasts. These cells were grown for 1–2 h. Purity of the erythroid cells from embryonic liver (85–90%) was determined by FACS analysis using a specific monoclonal antibody. Germ cells were prepared from the gonads of 12.5 d.p.c. or 13.5 d.p.c. embryos by antibody-directed flow sorting, as described<sup>26</sup>. Primitive type A and type A + type B spermatogonia were isolated from testes of 6- and 8-day-old mice by gradient sedimentation<sup>27</sup>. The purity of germ cells in the recovered populations was consistently more than 85%.

B cells were prepared by disruption of spleens isolated from the wild type, *iEκT<sup>-/-</sup>* (ref. 11) and *3κE<sup>-/-</sup>* (ref. 12) mice in phosphate-buffered saline (PBS) followed by gentle pipetting and centrifugation through PBS. B cells were stimulated by growing at 37 °C in RPMI medium containing 10% FCS, supplemented with 20  $\mu$ g ml<sup>-1</sup> lipopolysaccharide (LPS) for 3 days. The resulting population typically contained >90% positive B cells as demonstrated by flow cytometry analysis. Stromal cell/IL7-dependent cell lines, representing pro-B cells, were derived from fetal liver of wild-type mice, as described<sup>28</sup>. Pre-B cells were obtained from spleens of Rag<sup>-/-</sup> mice carrying a  $\mu$  transgene in which B-cell differentiation was arrested at the pre-B-cell stage<sup>29</sup>. T cells were isolated from the thymus and grown in the presence of ConA (2  $\mu$ g ml<sup>-1</sup>) for 3 days in culture.

### Fibroblast isolation and SV-40 transformation

Primary mouse ear fibroblasts were isolated from ROSA26 reporter mice<sup>17</sup>. The skin was gently peeled off individual ears to expose fibroblasts, which were rubbed vigorously on a 10-mm tissue culture dish with the aid of a sterile pipette. The fibroblasts were allowed to grow for 8–10 days. T-antigen transformation was carried out by infection with supernatant from producer cell line  $\psi$ 2-SV40 (ref. 30). Cells were passaged whenever they reached confluence, 3–4 times. Following passage, the SV40-transformed cells were subcloned by limiting dilution.

### Fluorescence *in situ* hybridization

All cells used for FISH analysis, including those taken from the gonads or from embryos, were first incubated in culture with  $3 \times 10^{-5}$  M BrdU for 1 h before collection, in order to enable us to provide a marker for identifying cells in S phase. Preimplantation embryos, as well as purified ICM, were collected and fixed to a poly-L-lysine slide (Sigma) by putting them into a drop of 0.01 N HCl, 0.1% Tween 20. In the case of zygotes, cells were placed on the slide at a density which allowed the observation of pronuclei pairs without interference from other nearby zygotes. After drying, the slide was washed, dehydrated, permeabilized and fixed. All other cells were treated with hypotonic KCl solution, fixed in methanolacetic acid (3:1) and dropped on slides, as previously described<sup>6</sup>.

FISH was performed as described previously<sup>14</sup>. Briefly, denaturation was carried out by incubation in 70% deionized formamide, 2  $\times$  SSC at 68 °C for 2 min, and then slides were dehydrated by a series of ice-cold ethanol washes (70, 90 and 100% for 5 min each). Cosmid or plasmid DNA was labelled by nick-translation, substituting dTTP (deoxythymidine triphosphate) with bio-16-dUTP or with digoxigenin-11-dUTP (Boehringer Mannheim). For detecting the  $\kappa$  chain gene in ear fibroblasts, DNA was labelled by nick translation (Amersham) with Cy3 dCTP. The critical size range of probe molecules (smaller than 500 base pairs (bp) and preferably 150–250 bp) was achieved by empirically varying the amount of DNaseI in the nick-translation reaction. Unincorporated nucleotides were separated from the probe DNA by centrifugation through 1-ml Sephadex G-50 columns (Boehringer Mannheim). Probe DNA (10–50 ng) was mixed with cot-1 (Life Technologies) (2–3  $\mu$ g) and sufficient salmon sperm DNA to obtain a total of 10  $\mu$ g in a 10- $\mu$ l hybridization cocktail. After denaturation of the probe mixture (80–90 °C for 5 min), pre-annealing of repetitive DNA sequences was carried out for 10 min at 37 °C before application to denatured nucleic-acid specimens. Following incubation overnight and subsequent post-hybridization washes, the specimens were treated with blocking solution (3% BSA, 4  $\times$  SSC) for 10 min at 37 °C in 1% BSA, 4  $\times$  SSC and 0.1% Tween 20 and slides were then washed at room temperature three times for 3 min each in 4  $\times$  SSC and 0.1% Tween 20. Biotin-labelled probes were detected with rhodamine-conjugated avidin DCS (1:500 dilution) (Vector Laboratories) and digoxigenin-labelled probes were detected with an anti-digoxigenin antibody conjugated to fluorescein isothiocyanate (FITC; Boehringer Mannheim) (1:100 dilution). BrdU was detected by anti-BrdU antibody (NeoMarkers) (1:100), followed by either rhodamine (1:50) or aminomethylcoumarin acetic acid (AMCA; 1:20) conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories). Counterstaining, where needed, was done using diaminodiphenylidole (DAPI) (200 ng ml<sup>-1</sup>) in Vector antifade solution. Amplification of the digoxigenin-labelled probes was carried out with anti-sheep antibody conjugated to FITC (Vector) and of the biotin-labelled probes with biotinylated anti-avidin (Vector). For the *neo-lacZ* sequence, amplification of the digoxigenin-labelled probe was done using three antibody layers, sheep anti-dogoxigenin (Roche), FITC-labelled rabbit anti-sheep (Calbiochem) and FITC-labelled goat anti-rabbit (Roche).

Replication timing profiles (per cent single/single, single/double and double/double) were determined by counting a minimum of 35–300 BrdU-positive nuclei for each cell type. The experiment involving zygote analysis was repeated over 15 times, obtaining 15–20 zygotes per female, thus enabling us to accumulate an average of 45 nuclei for each point on the graph.

The mouse probes were donated by and purchased from: B. Van Ness (pSP1gB, carrying the 12.0 kb *Bam*HI fragment encompassing the J-C $\kappa$  region and its derivative pGL1.6, representing the region upstream to J $\kappa$ 1); F. Sablitzky (pGLRIC $\mu$ , containing the 10.7-kb *Eco*RI fragment harbouring the C $\mu$  gene); H. Schroeder (pDFL3p8, containing a 5' 3.8-kb fragment upstream of DFL16.1 in the heavy-chain locus); J. Roess (p1g303A, containing a 6-kb fragment harbouring the CA1 region); J. Chen (pBL40.1, containing 9-kb *Eco*RI fragment located 3' to V $\beta$ 14 in the TCR $\beta$  locus); L. Jackson-Grusby (pSA $\beta$ geofrtPA, containing a 5.6-kb *Xho*I fragment carrying the  $\beta$ gal gene); D. Ward (chr 6, a random cosmid clone from chromosome 6); P. Fraser (mouse  $\beta$ -globin gene region plasmid p $\beta$ 12g and  $\beta$ major); N. Benvenisti (15-kb *TMP* (tumour-associated membrane protein) gene plasmid); I. Simon (CD2 gene-containing plasmid); H. Zachau (F1 cosmid DNA containing the 3' V $\kappa$  region); M. Reth (11.7-kb mouse *Rpi* gene pDR9 plasmid); F. Alt (4.11  $\phi$  DNA, containing 15 kb of genomic DNA located ~170 kb 3' to the C $\mu$  gene); Resgen (BAC 122K2, harbouring sequences located 2,000 kb upstream of C $\kappa$ ); Cloneteck (the random BAC clone, 212a06).

### Replication timing by S-phase fractionation

Abelson-virus-transformed pre-B-cell subclones from *M. spretus*  $\times$  *M. musculus* F<sub>1</sub> mice were labelled in 75  $\mu$ M BrdU for 45 min before collection, and nuclei were then sorted for cell-cycle fractions according to DNA content<sup>18</sup>. BrdU DNA was isolated from each fraction and assayed for specific sequence content by quantitative PCR carried out in two stages<sup>14</sup>, first for 18 cycles and then again for 10 cycles (95 °C 40 s, 55 °C 40 s, 72 °C 40 s) in the presence of [ $\alpha$ -<sup>32</sup>P] dCTP using the primer 5'-CAGACTGACCTCATGTCAGA-3' (170-1) together with 5'-AATGAGCAAAGTCTACTTACG-3' (K-2) or 5'-CAGAACCAAAAGTCAACAAGTA-3' (K-4) for mouse  $\kappa$ . PCR products from the S-phase fractions were electrophoresed after cutting with *Hha*I (present only on the *M. musculus* allele) and detected by autoradiography. In order to graph each allele separately, total uncut PCR

product was first quantified on an initial gel. The proportion of maternal or paternal alleles in each lane was then determined from a second gel after cutting the PCR product. These values were obtained by scanning the autoradiographs.

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**Identification of the cellular receptor for anthrax toxin**

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**The tripartite toxin secreted by *Bacillus anthracis*, the causative agent of anthrax, helps the bacterium evade the immune system and can kill the host during a systemic infection. Two components of the toxin enzymatically modify substrates within the cytosol of mammalian cells: oedema factor (OF) is an adenylate cyclase that impairs host defences through a variety of mechanisms including inhibiting phagocytosis<sup>1,2</sup>; lethal factor (LF) is a zinc-dependent protease that cleaves mitogen-activated protein kinase kinase and causes lysis of macrophages<sup>3–5</sup>. Protective antigen (PA), the third component, binds to a cellular receptor and mediates delivery of the enzymatic components to the cytosol. Here we describe the cloning of the human PA receptor using a genetic complementation approach. The receptor, termed ATR (anthrax toxin receptor), is a type I membrane protein with an extracellular von Willebrand factor A domain that binds directly to PA. In addition, a soluble version of this domain can protect cells from the action of the toxin.**

After binding to the cell-surface receptor, PA is cleaved into two fragments by a furin-like protease<sup>6</sup>. The amino-terminal fragment, PA<sub>20</sub>, dissociates into the medium, and this allows the carboxy-terminal fragment, PA<sub>63</sub>, to heptamerize and to bind LF and OF<sup>7,8</sup>. The resulting complexes of [PA<sub>63</sub>]<sub>7</sub> with OF and/or LF are taken up into cells by receptor-mediated endocytosis and moved to a low-pH endosomal compartment<sup>9</sup>. There, the acidic environment induces a conformational change in [PA<sub>63</sub>]<sub>7</sub> that allows it to insert into the membrane and form a pore<sup>10–12</sup>. This conversion promotes the translocation of bound OF and LF across the endosomal membrane to the cytosol.

Previous studies have indicated that the receptor to which PA binds is a ubiquitous protein expressed at moderately high levels on cell surfaces (for example, 10<sup>4</sup> and 3 × 10<sup>4</sup> receptors per cell on CHO-K1 cells and macrophage cell lines, respectively)<sup>13,14</sup>. To identify this receptor, we first generated a mutant cell line lacking receptor, so that the defect could be genetically complemented. ICR-191, a DNA alkylating agent that induces small deletions and frameshift mutations in genes<sup>15</sup>, was used to introduce random mutations in the hypodiploid CHO-K1 cell line under conditions that led to about 90% cell death. The surviving mutagenized cells were then challenged with PA and LF<sub>N</sub>-DTA, a fusion protein composed of the N-terminal 255 amino acids of LF linked to the catalytic A chain of diphtheria toxin<sup>16</sup>. This recombinant toxin can kill CHO-K1 cells (in contrast to LF and PA) and it exploits the same LF-PA-receptor interactions that are required for the binding and entry of the native LF and OF proteins. Ten single-cell colonies (designated as CHO-R1.1 to CHO-R1.10) that survived toxin treatment were isolated. In control experiments performed with non-mutagenized CHO-K1 cells, no toxin-resistant cell clones were detected. One of the mutagenized clones (CHO-R1.1) was chosen for further analysis.

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