Pergamon

European Journal of Cancer Vol. 33, No. 5, pp. 792–800, 1997

Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(97)00060-9

Telomere Structure and Telomerase Expression During Mouse Development and Tumorigenesis

D. Kipling

Department of Pathology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

Mouse telomeres are on average longer than those of man, raising questions regarding the link between telomere loss and replicative senescence in mice and the requirement for telomerase activity for mouse cell immortalisation. However, the emerging data on telomerase activity during tumorigenesis in the mouse must be interpreted in the context of the very different structure of mouse telomeres. It will be argued here that the evidence for a casual link between telomere loss and replicative senescence is weak in the mouse, with the observed upregulation of telomerase activity in mouse tumours perhaps instead reflecting co-ordinated regulatory changes in tumour cells. Its absence would be consistent with evolutionary considerations, which hypothesise that such a link is an additional layer of control against tumour formation that has evolved in man. The very different genomic substrates for telomerase in humans and mice mean that the initial phenotype of a telomerase knock-out mouse does not necessarily critically address the existence of a link between telomerase and tumorigenesis in man.

Key words: telomere, telomerase, senescence, immortalisation, mouse

Eur J Cancer, Vol. 33, No. 5, pp. 792–800, 1997

INTRODUCTION

THE MAJORITY of studies relating telomerase activity and telomere length to immortalisation and neoplasia has centred on human cells (reviewed in [1, 2] and elsewhere in this issue). Increasingly, however, the laboratory mouse is being used as a potential model system to experimentally investigate these phenomena, offering as it does both powerful genetics and the possibility of direct interventional studies by targeted gene disruption. Telomerase activity can be detected in the mouse and the cloning of its RNA component will now enable its gene to be deleted in transgenic mice. Such a knock-out mouse, which in principle should not show telomerase activity in any tissue, will be of interest with respect to its viability, breeding, stem cell behaviour, haematopoietic system and of course cell senescence and immortalisation. It has the potential to shed much light on the role of telomerase in these processes. It is important, however, that it be borne in mind that telomerase does not work alone, but rather functions in consort with the $(TTAGGG)_n$ terminal repeat sequences. Differences in telomere structure between species could lead to wildly different outcomes should telomerase be absent. It is, therefore, vital that any interpretations regarding the role of telomerase in tumorigenesis be made in the context of a thorough understanding of the data surrounding mouse telomere structure, and not simply be used to extrapolate to the situation in human cells. This review aims to describe in some detail what is currently known about mouse telomere structure and to incorporate recent data on telomere dynamics and telomerase expression during mouse development and tumorigenesis. To conclude, some predictions will be made regarding the possible phenotypes of a transgenic mouse lacking telomerase.

MOUSE TELOMERES ARE ON AVERAGE LONGER THAN IN MAN

Human germ-line telomeres consist of around 15–25 kb of terminal $(TTAGGG)_n$, and can be visualised as a smear on Southern blots following digestion of genomic DNA with restriction enzymes that cut frequently in the genome (see the article in this Special Issue by T.M. Bryan and R.R. Reddel, pages 767–773). In contrast, most mouse telomeres appear much longer. For example, digestion of DNA from the *Mus musculus* inbred laboratory strain DBA/2 with the frequent-cutting restriction enzyme *Mbo*I releases $(TTAGGG)_n$ restriction fragments 20–150 kb in size, many of which can be resolved as discrete fragments on pulsed field gels [3, 4]. Such an analysis is shown in Figure 1, where DNAs from a number of mouse cell lines and animals are shown digested with *Mbo*I and hybridised with a probe to detect $(TTAGGG)_n$. Lane 7 shows a sample from the inbred *M. musculus* laboratory strain CBA/Ca, and it is clear that in many cell lines a similar size-range of teminal restriction fragments is maintained.

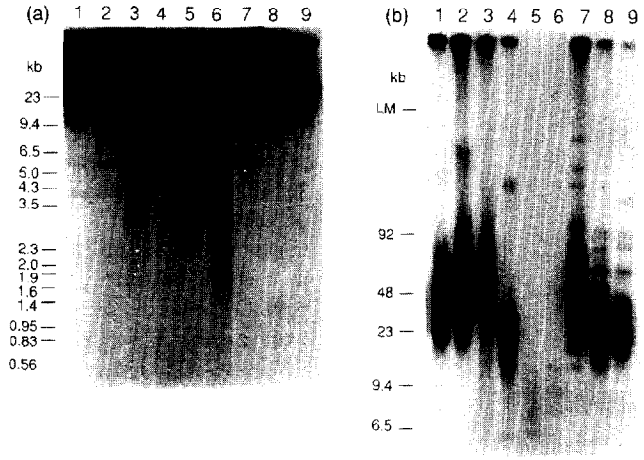

Figure 1. $(TTAGGG)_n$ arrays in mouse cell lines and in two mouse species. Genomic DNA was digested with *Mbo*I, separated by conventional (a) or pulsed field (b) gel electrophoresis and filters probed with a $(TTAGGG)_4$ oligonucleotide. Samples shown are from a F9 teratocarcinoma cell line (lane 1), ES cell line (lane 2), X63/NS1 mouse myeloma cell line (lane 3), L929 mouse fibroblast cell line (lane 4), C127 mouse mammary tumour cell line (lane 5), RAG mouse renal adenocarcinoma cell line (lane 6), CBA/Ca female mouse spleen (lane 7), *M. spretus* female spleen (lane 8) and *M. spretus* male spleen (lane 9).

Are mouse telomeres really composed of extensive terminal repeat arrays, or is there some other explanation? A number of possible genomic organisations, which would produce such data, are shown in Figure 2a–d. Firstly, all telomeres in the cell could have $(TTAGGG)_n$ arrays around 50 kb in size (Figure 2a). Secondly, mouse telomeres could be short with this signal obscured by large interstitial $(TTAGGG)_n$ arrays in the genome (Figure 2b). Thirdly, the size of the restriction fragments released by *Mbo*I could be large for all telomeres, but would consist predominantly of some other non-telomeric simple sequence with only a small cap of $(TTAGGG)_n$ (Figure 2c). Finally, most telomeres could have large $(TTAGGG)_n$ arrays with one or two telomeres maintained at a substantially shorter length (Figure 2d). It will be argued below that the data most strongly support the first and fourth models, and a possible mechanism to allow for the stable biphasic organisation shown in Figure 2d will be suggested.

The first line of evidence that mouse telomeres are in fact composed of long, uninterrupted arrays of telomeric repeats is the amount of $(TTAGGG)_n$ in mouse cells. This has been estimated by quantitative hybridisation to be around 10-fold more than in human cells [3, 4]. This is what is expected if the terminal repeat arrays are indeed as long as they appear and argues against the 'small cap' model (Figure 2c). However, maybe most of the $(TTAGGG)_n$ in the mouse is at non-telomeric locations (Figure 2b). This does not appear to be the case, since most $(TTAGGG)_n$ in the mouse is exonuclease BAL31 sensitive [3, 4], indicating that the $(TTAGGG)_n$ signal seen on Southern blots is mainly associated with terminal restriction fragments. Another line of evidence in support of a model where most telomeres are composed of large $(TTAGGG)_n$ tracts comes from the amount of $(TTAGGG)_n$ added during telomere-associated chromosome fragmentation experiments (reviewed in [5]). In mouse embryonic stem cells, around 50 kb of $(TTAGGG)_n$ is added to introduced plasmids terminating in short $(TTAGGG)_n$ arrays, considerably more than is added to such constructs in human cell lines [6]. This suggests that optimal telomere length is set at a relatively high value in this totipotent primary mouse cell.

Mouse $(TTAGGG)_n$ arrays are very dynamic structures. The $(TTAGGG)_n$ signal can be resolved into a number of discrete fragments by pulsed field gel electrophoresis (PFGE). These differ from mouse to mouse of the same inbred strain, and new variants readily occur in family studies [3, 4]. There is little precedent for an internal genomic sequence in the mouse showing this degree of hypervariability and sequence alteration, but if these are terminal $(TTAGGG)_n$ fragments then this hypervariability can be readily accounted for by the dynamics of telomere turnover [7]. That is, during cell division the germ line telomeres are subject to a continual process of sequence loss (because of the end replication problem) and sequence addition (by telomerase and possibly other mechanisms). The diploid zygote, where there are two copies of each telomere, gives rise to an adult mouse whose germ line now contains many cells, each with telomeres slightly different in length. Reproduction leads to the selection of a single cell from the germ line and thus selection of a single telomere length from the range of sizes available. From generation to generation it is easy to imagine how telomere length could drift slightly, leading to the variability observed. In a somewhat similar manner, the size of these large $(TTAGGG)_n$ fragments can be observed to change during continued culture and clonal selection of mouse cells *in vitro* [8]. This again can most easily be explained if these fragments are terminal. Therefore, the expected dynamic behaviour of mouse telomeres predicts the hypervariability seen.

Are there many internal $(TTAGGG)_n$ arrays in the mouse which might confuse analysis? The hypervariability of the $(TTAGGG)_n$ signal suggests not, and indeed very few internal arrays have been detected. None is detected by the relatively insensitive technique of *in situ* hybridisation [2] although some very short and somewhat degenerate arrays have been cloned from the mouse and mapped [9–11], one of which may correspond to what in humans is the telomere of chromosome 16p [12].

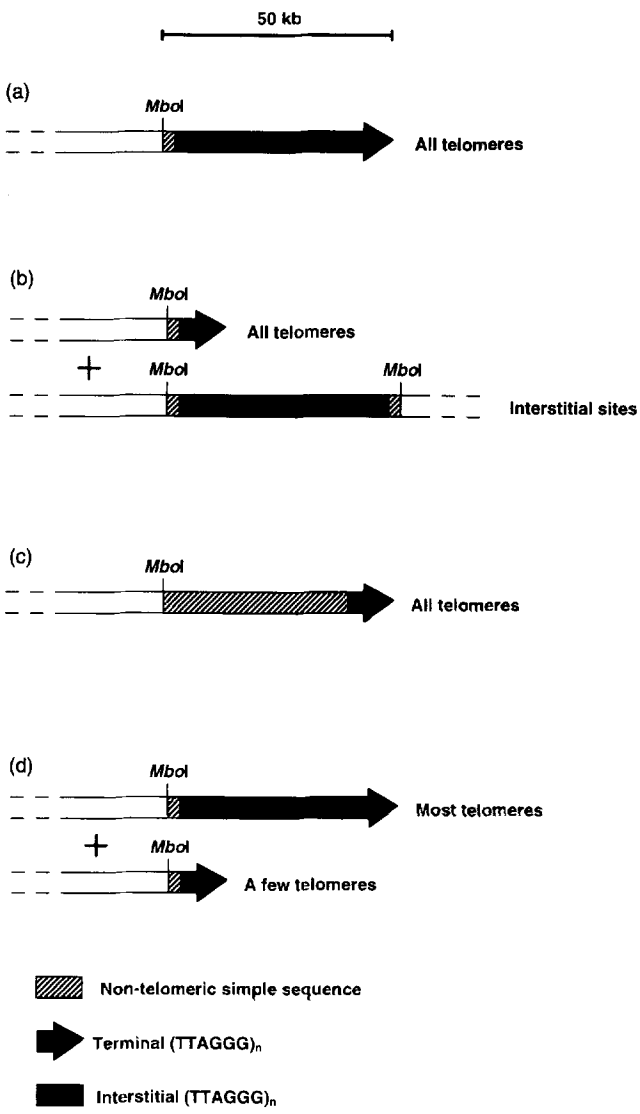

Figure 2. Models for mouse telomere structure. In each model the size of the terminal (TTAGGG)$_n$ array (black arrow), interstitial (TTAGGG)$_n$ (black rectangle), and the nearest *Mbo*I site is shown. In humans a small amount of non-(TTAGGG)$_n$ sequence is included on such terminal restriction fragments, and by analogy a similar amount is shown here (hatched rectangle) except for (c) where the amount is much greater. See text for further details.

Some interesting data regarding telomere structure come from an unusual colony of *Mus spretus* held in the U.S.A. such as the animals available from The Jackson Laboratory. Starling and associates [4] originally demonstrated that these U.S. animals contain a much reduced total amount of (TTAGGG)$_n$ in their genome, together with much smaller (TTAGGG)$_n$ restriction fragments, a situation that enabled them to be resolved by conventional agarose gel electrophoresis. Prowse and Greider [13] also demonstrated that there are relatively few exonuclease BAL31-insensitive fragments in the U.S. *M. spretus*, all of which are modest in size. Thus this species does not have large interstitial arrays of (TTAGGG)$_n$.

The U.S. colony of *M. spretus*, which may have come from a relatively small number of animals originally exported to the U.S.A., is very different from the animals captured and maintained in Europe. (TTAGGG)$_n$ fragments from two such animals are shown in Figure 1b (lanes 8, 9) and are in the 20–100 kb size range. The intensity of the (TTAGGG)$_n$ signal, normally much weaker in *M. spretus*, is now comparable to that seen for the CBA/Ca sample shown in lane 7. It is not clear what has caused the U.S. population of *M. spretus* to have such small telomeres. Again, this is most readily explained by the dynamic nature of telomeres and their length drifting over time, perhaps compounded by mutations affecting telomere length arising in a small founder population and becoming fixed in this partially inbred colony. Irrespective of the details of the mechanism, it seems unlikely that the difference in the (TTAGGG)$_n$ signal between the two colonies reflects dramatic changes in the amount of interstitial (TTAGGG)$_n$ in the genome. The American *M. spretus* colony therefore provides strong circumstantial evidence that there is little interstitial (TTAGGG)$_n$ in the mouse genome. It should be stressed that these animals are somewhat atypical. All other laboratory mouse strains analysed so far show the long telomeres originally described for DBA/2 and C57BL/6, and it is this genome organisation which appears to be nearer the situation in natural populations, as *M. musculus* animals caught in the wild have telomeres substantially longer than those of the American *M. spretus* [14].

The data are most consistent with the majority of the (TTAGGG)$_n$ in the mouse genome being located at the end of the chromosomes, behaving in a manner consistent with the expected dynamic turnover of terminal (TTAGGG)$_n$ arrays. As most of the (TTAGGG)$_n$ appears terminal, an inescapable conclusion from the relative amounts of total (TTAGGG)$_n$ in humans and mouse is that the majority of the length of the large (TTAGGG)$_n$ fragments seen by PFGE is indeed this sequence.

TELOMERE DYNAMICS IN CELL LINES AND TUMOURS

Although the telomere structure of the American *M. spretus* animals is somewhat atypical, it has enabled telomere loss rates of *in vitro* cultured cells to be measured [13]. A shortening rate of 75 bp/division was observed, similar to that seen for human telomeres in cultured cells. It was argued above that a model where most telomeres contain large (approximately 50 kb) stretches of terminal (TTAGGG)$_n$ best fits the available data. Such a model makes a number of predictions regarding the dynamics of telomere structure in cell lines, with age and in tumours.

If most of the (TTAGGG)$_n$ is terminal it should be possible to create a cell line with very small amounts of (TTAGGG)$_n$. Consider the extreme situation where end replication losses during establishment of the cell line remove most of the terminal (TTAGGG)$_n$ before telomere length stabilises. In the 'small cap' model (Figure 2c) this would result in the (TTAGGG)$_n$ signal being very weak but still on large restriction fragments, whereas in the interstitial (TTAGGG)$_n$ model (Figure 2b) the overall signal would be largely unchanged. As shown in Figure 1, some mouse cell lines do show very weak (TTAGGG)$_n$ signals, such as C127 and RAG (lanes 5 and 6). The reduction in (TTAGGG)$_n$ signal is always accompanied by a dramatic decrease in the size of the terminal restriction fragments. The existence of such cell lines is most consistent with a model of telomere structure where most of the terminal

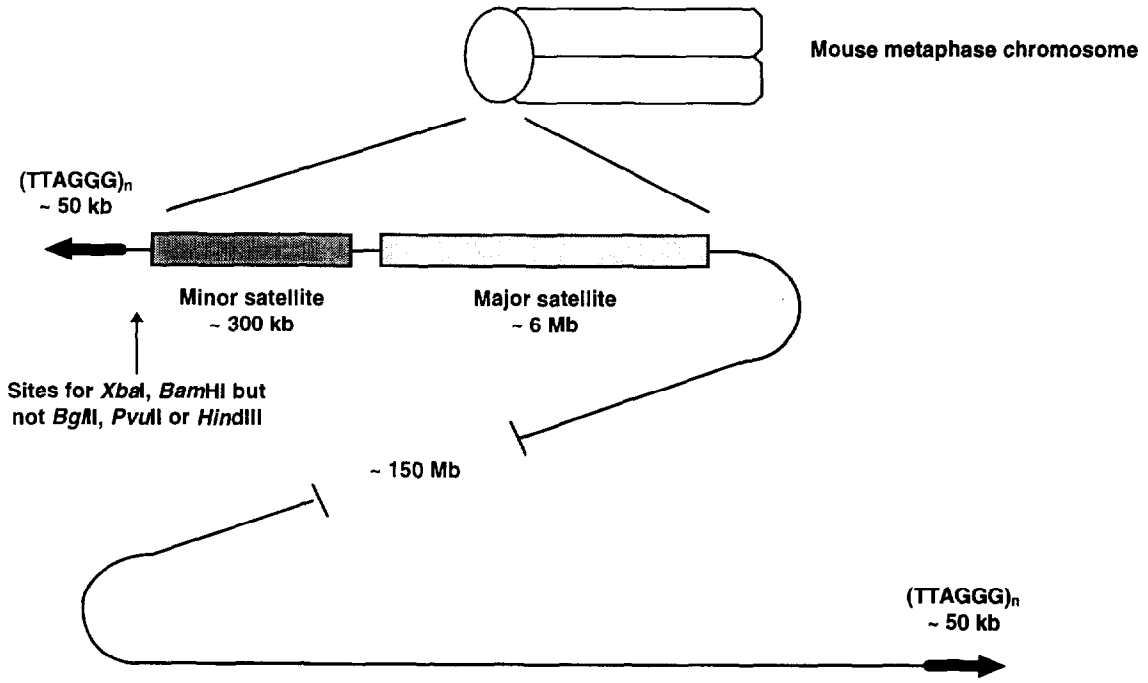

Figure 3. Schematic representation of the hypothetical sequence organisation of a 'typical' *Mus musculus* autosome. The minor satellite co-localises with the kinetochore and is a good candidate for a functional centromeric sequence, whereas the major satellite underlies the pericentric heterochromatin. See text for further details.

fragments are composed of large $(TTAGGG)_n$ arrays (Figure 2a or d).

The large size of mouse telomeres (approximately 50 kb) plus the rate of telomere loss (75 bp/division, assuming the somewhat unusual *M. spretus* colony in the U.S., provides a reliable estimate for the telomere loss rate in *M. musculus*) allows some very rough estimates to be made for telomere dynamics during the growth of animals and also during cell immortalisation *in vivo* and *in vitro*. Forty population doublings would lead to the loss of $40 \times 75 = 3$ kb of $(TTAGGG)_n$. It would be difficult to detect convincingly such a small change in a 50 kb restriction fragment by PFGE. Thus, a large number of cell divisions could occur during the life of a mouse without producing a dramatic change in telomere size between old and young mice, and indeed no such marked change is seen [3, 4]. Similarly, despite a large number of cell divisions, telomere size changes in tumours would be difficult to detect with this amount of sequence loss, and indeed initial data suggest that mouse tumours show very similar telomere sizes to surrounding tissue [8, 15].

What is crucial to bear in mind is that the relationship between telomeres and cancer or cell senescence does not require *all* telomeres to be short. It is hypothesised that the cell is measuring the length of the shortest telomere as the important variable. Thus, if mouse telomeres are *on average* longer than those in the human this may not be relevant. What is important is the size of the shortest telomere in a mouse, and its relationship to the size of the shortest telomere in a human cell. The PFGE data indicate that mouse telomeres start at around 20 kb in size (see Figure 1; lanes 7–9) and thus the bottom of the distribution of telomere sizes in the mouse overlaps the size of telomeres in human germ-line tissue.

SUBTELOMERIC REPEATS

Relatively little is known about the sequences adjacent to the terminal $(TTAGGG)_n$ repeats in the mouse. Mouse chromosomes (except the Y) are telocentric, with the centromere at the very end of the chromosome (Figure 3). The current best candidate functional sequence for the *M. musculus* centromere is the minor satellite DNA [16]. This may be functionally equivalent to primate alphoid DNA in that both possess functional 17 bp binding sites for the centromere-associated protein CENP-B [16]. The minor satellite is composed of tandem repeat arrays of an underlying 120 bp monomer unit, these arrays ranging in size from several hundred kilobases to over a megabase [17]. Being composed of relatively simple sequence DNA, they do not contain restriction sites for most enzymes with a 6 bp recognitiion sequence, and these enzymes can therefore be used to cleave and release the array on each chromosome largely intact. PFGE data using minor satellite and $(TTAGGG)_n$ indicate that in digests such as *Bgl*II, *Hin*dIII or *Pvu*II, there are, in addition to the expected $(TTAGGG)_n$ signal in the 20–100 kb range, a number of discrete fragments ranging from 400 kb to over 1.5 Mb [17]. All of these unusually large $(TTAGGG)_n$ fragments also hybridise to minor satellite. The conclusion is that the minor satellite is so close to the end of the chromosome that there are rarely sites for these enzymes between the minor satellite and $(TTAGGG)_n$ — the two sequences are on the same, terminal restriction fragments (see Figure 3). The absence of these three enzyme sites suggests that there is little if any of the complex DNA typical of euchromatic regions between this candidate centromeric sequence and the telomere, and that there are likely to be few, if any, genes between mouse telomeres and centromeres. Mouse chromosomes seem truly telocentric.

One relevant point is that for some enzymes, such as *Bam*HI or *Xba*I, no physical linkage of minor satellite and $(TTAGGG)_n$ is observed [17]. Instead, all the $(TTAGGG)_n$ signal is in the 20–100 kb range [17]. The published PFGE analyses [3, 4] where telomere length in the mouse was measured use the restriction enzyme *Mbo*I. This recognises a GATC motif that is contained within all *Bam*HI sites. Because of the lack of physical linkage of $(TTAGGG)_n$ and minor satellite in *Bam*HI digests, there will be no minor satellite included on the *Mbo*I fragments used to determine telomere length. The measurement of mouse telomere length using *Mbo*I is therefore not complicated by the presence of minor satellite on the terminal restriction fragments.

The PFGE analyses suggested the presence of a common subtelomeric structure for mouse centromeric telomeres. Most proximal telomeres show an absence of *Bgl*II, *Hin*dIII and *Pvu*II sites between the minor satellite and the $(TTAGGG)_n$, whereas almost all contain *Bam*HI and *Xba*I sites [17]. One possible explanation for this apparently similar subterminal restriction map is the existence of a repeated sequence in the subterminal region of all these telomeres containing or lacking the appropriate restriction sites. One such candidate is the sequence family detected by the ST-1 probe [18]. This 670 bp sequence was initially identified by PCR amplification using $(TTAGGG)_n$ oligonucleotides [18]. By tritium *in situ* hybridisation ST-1 was localised to all mouse telomeres, both proximal and distal. ST-1 may be part of a much larger region of shared homology between telomeres but it is provocative that the published sequence contains a *Bam*HI site [18], suggesting that ST-1 and adjacent sequences may well be responsible for the apparent similarities in restriction maps between mouse telomeres.

Mapping mouse telomeres by providing genetic and sequence tagged sites (STSs) to define the ends of the genetic and physical maps of mouse chromosomes is an important part of the mouse genome project. Some proximal telomeres can be mapped using variation between strains in the size of the terminal restriction fragments on PFGE detected by either $(TTAGGG)_n$ or oligonucleotides which detect subsets of the minor satellite arrays [16, 17, 20]. On conventional agarose gels, RFLPs (restrictive fragment length polymorphisms) detected by a $(TTAGGG)_n$ probe have been mapped to a number of telomeres including those of the distal X, Y, 4, 9 and the short arm of the Y [21, 22]. Some success has been obtained cloning mouse telomeres using the 1/2-YAC technology used initially to clone human telomeres [23]. Clones exist for the distal telomere of chromosome 10 [19] as well as the region adjacent to the $(TTAGGG)_n$ array in the pseudoautosomal region [9, 24]. The latter region contains sequences also present at the distal telomeres of mouse chromosomes 4, 9 and 13, these telomeres forming a region of generalised similarity that extends to other mouse species such as *M. spretus*, *M. musculus molossinus*, *M. musculus castaneus*, *M. spicilegus* and *M. hortulanus* [24, 25], suggestive of ancestral telomere–telomere duplication and exchange reactions. In a separate study, Rounds and associates [26] used an affinity capture protocol to identify a number of cosmids containing telomere-proximal sequences, including clones specific for the distal telomeres of chromosomes 3, 4, 5 and 12.

TRF AND TELOMERASE IN THE MOUSE

Mouse telomeres have a size and genetic behaviour somewhat different from human telomeres, which raises questions as to the extent to which telomere dynamics are similar in the two species. A number of factors might cause such differences. Telomeres do not exist in a vacuum, but are part of a nucleoprotein complex and interact with other proteins such as telomerase. In mammals, one of the protein components of the telomere is telomere repeat factor (TRF), which has recently been cloned in the mouse [27]. The RNA for this protein is ubiquitously expressed and the protein, when synthesised *in vitro*, appears to have the same binding characteristics as human TRF. However, despite two well-conserved domains, the overall amino acid identity between human and mouse TRF is only 67%. Although no further data exist on this point, the differences in TRF sequence between the two species open one route to allow species-specific differences in telomere dynamics, especially if the qualitative or quantitative aspects of TRF function can modulate telomere length regulation or telomerase accessibility. Another difference between the two species is that under standard conditions mouse telomerase is non-processive *in vitro* as compared to the human enzyme [28]. The reason for this difference in telomerase behaviour is unknown. The RNA component of mouse telomerase has a shorter templating region and this has been suggested [29] as one potential explanation for the lack of processivity of the mouse enzyme, as it may allow the enzyme to dissociate more readily between rounds of extension. The human enzyme produces a ladder of products on a sequencing gel, whereas the mouse enzyme produces a single predominant band. However, under conditions of high dGTP the mouse enzyme will also produce a ladder of extension products [30]. The protein components of human and mouse telomerase have not been cloned and it will be interesting to discover their similarity.

One crucial question that has not been addressed is whether there is also a difference in the size of a telomere recognised as 'critically short' between human and mouse. Mouse telomeres appear somewhat larger, which might suggest that many more divisions would be required before a sufficiently short telomere was formed to signal cell cycle arrest. However, there are in fact few data to suggest that the 'critical telomere size' is the same in human and mouse. Perhaps the only evidence that a 30 kb telomere in the mouse does not signal cell cycle arrest is the viability of the American *M. spretus* colony. This is clearly a point which deserves further attention.

TELOMERASE ACTIVITY DURING DEVELOPMENT, DIFFERENTIATION AND MULTI-STAGE TUMORIGENESIS

Telomerase activity is a very good marker for tumorigenesis in man (reviewed in [1] and see the article in this Special Issue by N. Kim, pages 781–786), as it is detectable in very few somatic human tissues and is upregulated in a large proportion of tumours. In contrast, the regulation of mouse telomerase appears at first sight to be much less stringent, with many primary tissues of both laboratory mice and *M. spretus* expressing detectable amounts of telomerase.

Telomerase can be detected in the mouse both by the conventional assay and the more sensitive TRAP protocol. With both of these it must be borne in mind that *in vitro* ac-

tivity is being measured, and this does not necessarily predict the *in vivo* activity. Furthermore, it is possible that low but detectable levels of telomerase can be present without adding sufficient *de novo* telomere sequence to compensate for that lost because of the end replication problem. Thus, a cell could in theory express detectable quantities of telomerase, but still show telomere shortening with continued cell division. Such caveats are important for both mouse and human studies of the relationship between telomerase, telomere loss and tumorigenesis.

A number of studies have detected telomerase activity in a wide range of primary somatic tissue in the mouse [13, 31]. Tissues from both standard laboratory mice and *M. spretus* that contain detectable telomerase include skin, lung, liver, colon and uterus [13, 31]. Interestingly, many of the telomerase-negative tissues such as brain and muscle contain largely non-dividing cells. Indeed, explants of skin and mammary tissue show little telomerase activity initially, but after only a short time of *in vitro* culture telomerase activity increases considerably [31]. This suggests that mouse telomerase is subject to control relative to the proliferative state of the cell.

Although telomerase activity is present throughout the cell cycle of both mouse and human cells [30], mouse telomerase activity is downregulated strongly upon exit from the cell cycle into a G_0-like quiescent state. A number of *in vitro* culture systems illustrate this. NIH 3T3 cells become quiescent upon contact inhibition, entering a G_0 state. This quiescence is accompanied by a very dramatic downregulation of the level of telomerase activity, an effect which is reversible by reseeding cells at lower density and allowing proliferation to resume [32]. Similarly, C2C12 mouse myoblasts differentiate to form myotubes upon growth factor removal, this again being accompanied by downregulation of telomerase activity. Leucocytes are one of the few human somatic cells that express detectable quantitites of telomerase, and for this naturally quiescent cell type a marked upregulation of telomerase activity has been noted upon entry into the cell cycle following stimulation with phytohaemagglutinin or antibodies to the T-cell receptor [33]. Furthermore, both mouse embryonic stem (ES) and embryonal carcinoma (EC) cells differentiate *in vitro*, by LIF (leukaemia inhibitory factor) removal and retinoic acid addition, respectively, a differentiation that is accompanied by a dramatic reduction in telomerase activity [34]. In these examples it is not totally clear whether differentiation *per se*, in the absence of a reduction of proliferation, has an effect on telomerase activity, as differentiation is usually accompanied by entry into a postmitotic state. The effect of proliferative state on telomerase activity must be considered when interpreting data from mouse tumorigenesis, as late-stage tumours are often marked by a high proliferative index.

Telomerase and telomere length have been analysed in a number of model systems of multistage tumorigenesis. Chadeneau and associates [31] analysed transgenic mice expressing high levels of the *neu* gene transcribed from the MMTV promoter/enhancer in mammary tissue. Such mice develop telomerase-positive mammary tumours and lung metastases. However, telomerase activity was also detected in other tissues, even in the parental non-transgenic strain. Blasco and colleaguges [35] have studied two transgenic mouse models of multistage tumorigenesis. The first are mice expressing part of HPV16 in basal keratinocytes, resulting in multistage induction of squamous cell carcinomas. Although a relatively small number of samples were analysed and no telomere length measurements made, it appeared that there was a strong upregulation of telomerase in the very late stages of tumour formation, with much less telomerase induction in the earlier stages. Islet carcinomas of mice expressing SV40 T antigen in pancreatic β cells also showed an upregulation of telomerase compared with normal islets. In both cases the extent of cell proliferation in the various samples is not given, making it hard to dissociate effects of cell proliferation from other alterations in tumour cells. Bednarek and associates [36] have analysed mouse skin papillomas induced by chemical treatment and report a strong upregulation of telomerase in the aneuploid, highly dysplastic late-stage diploid papillomas, despite roughly similar proliferative indexes. Finally, Broccoli and colleagues [15] have studied in detail another multistage tumorigenesis system, that of mammary tumours caused by expression of the *Wnt-1* oncogene from the MMTV promoter. They observed upregulation of telomerase in tumours as opposed to normal mammary tissue or hyperplastic tissue, an effect independent of the p53 genotype.

What happens to telomere length in mouse tumours? Initial data [8] revealed little if any difference in the telomere sizes between tumour and normal tissue, and this has been confirmed in subsequent studies [15]. Indeed, the individual-specific $(TTAGGG)_n$ banding pattern is identical in the tumours, which argues strongly that the tumour cells have not undergone dramatic telomere loss followed by telomere growth during the cell division leading to the formation of the tumour. Rather, it seems likely that the telomere length has stayed largely unchanged during this process.

In all these studies the crucial question is why is telomerase upregulated? As argued above, during the cell divisions leading to formation of the tumour there does not seem to have been a period where the telomeres became very short. Without this occurring, what selective pressure could there be to upregulate telomerase? If telomere loss is not the driving force, what is? One possibility is that cell proliferation *per se* is the main determinant of telomerase expression in the mouse, to which end it is important to correlate the extent of cell proliferation in mouse tumours with levels of telomerase activity. Broccoli and associates [15] showed that the level of telomerase RNA strongly correlates with histone H4 mRNA in all tissues, suggesting that it closely follows proliferative state. They also showed that telomerase activity is more greatly upregulated in tumours than is the RNA component, indicating that although telomerase is proliferation-sensitive in normal tissues, there are additional factors which further upregulate it in tumours. Whether this additional layer of regulation involves transcriptional or post-transcriptional regulation (e.g. protein phosphorylation) remains to be determined.

In a very broad sense, an immortal cancer cell is returning to a more primitive, ancestral phenotype. Senescence of somatic cells is dominant in cell fusion experiments, indicating that mortality is a gain of function. To create an immortal cancer cell may require a co-ordinated series of changes removing these levels of 'mortality control', one of which may be the removal of telomerase repression in somatic tissue. Although the end-point is very similar in that telomer-

ase provides an excellent marker for a tumour cell, the underlying reasons are very different. At one extreme telomerase expression is a necessary requirement for continued growth of the tumour cell, whereas at the other it is simply a side-effect of the co-ordinated gene expression changes which occur in tumour cells. It is also possible that the loss of 'mortality control' leading to telomerase upregulation, while it may not be strictly required, may provide a growth advantage that facilitates tumour formation and progression. Clinically, this is a very important point to address, as it has obvious implications for the use of antitelomerase drugs as potential antitumour therapeutics. However, care must be taken when extrapolating from the mouse to humans, as it is entirely possible that a requirement for telomerase in tumorigenesis exists in humans but not in mice.

REPLICATIVE SENESCENCE AND MOUSE TELOMERES

It is unclear whether replicative senescence in the mouse involves telomeres. The widespread presence of telomerase in proliferating tissue, together with the size of mouse telomeres and the measured shortening rate, at first make it seem somewhat unlikely that telomere loss could be the trigger for the early arrest seen for primary mouse fibroblasts, which stop dividing after only 10–20 population doublings in culture. However, there is no *a priori* reason to expect the mechanism of replicative senescence to be conserved throughout all mammalian cells. It is possible that some other factor, such as accumulation of non-specific DNA lesions caused by oxidative damage [37], is rate-limiting for mouse cell division. It is intriguing in this respect that DNA repair is a good marker for replicative potential in culture and that at least one enzyme related to DNA repair (PARP) shows activity proportional to the mean life span of a variety of mammalian species (reviewed in [38]). Even if telomeres are the rate-limiting factor for primary mouse cell division in culture, the link between replicative senescence and organismal ageing is far from clear, even in those tissues where cell division continues throughout the life of the organism [39]. Furthermore, even should a link be proved for humans, the situation need not be the same in the mouse.

As has been mentioned by a number of authors [31, 40], it seems likely that telomere loss may act as a way of suppressing tumour formation in humans. There is every possibility that this extra layer of protection is not conserved between all mammals. Repression of telomerase in somatic cells is stringent in humans but much less tight in the mouse. Indeed, the idea that the mouse lacks this method of reducing tumour incidence fits nicely with the ease with which mouse cells spontaneously immortalise. Human cells are, in comparison to mouse, some 10^6-fold more resistant to immortalisation [41]. The subsequent increased susceptibility of mice to cancer may have little impact on an animal that lives for such a short period of time.

DO MOUSE CELLS REQUIRE TELOMERASE FOR TELOMERE MAINTENANCE?

Mouse telomeres are marked by their great size and by length changes from generation to generation. Are there examples of human cells which mimic aspects of this behaviour? Bryan and associates ([42] and see the article in this Special Issue by T.M. Bryan and R.R. Reddell, pages 767–773) have analysed a number of SV40 immortalised human cell lines which are telomerase-negative, yet upon passage do not show telomere shortening. The consistent feature of these cell lines is that their telomere lengths are all very large (>23 kb). How telomere sequence addition occurs in such cells is unclear, but could involve a process permitted by their unusually large size, perhaps some form of strand slippage or gene conversion mechanism. A telomerase-independent mechanism of sequence addition to the ends of chromosomes can occur in budding yeast, specifically in *est1Δ* or *tlc1Δ* survivors. Here the chromosomes terminate in amplified arrays of the subtelomeric Y′ element, and a recombination-based mechanism causes further sequence addition to the ends [27, 43]. If such a large repetitive array in yeast can cause net sequence addition to chromosome ends via a recombination pathway, it is formally possible that a similarly large array of another sequence such as $(TTAGGG)_n$ could elicit a similar response. The fact that cell lines with telomeres similar in size to those of mice can be maintained in the absence of telomerase raises the obvious question of whether mouse telomeres utilise a similar telomerase-independent mechanism of sequence addition.

If this alternate pathway of telomere addition acts preferentially on very long telomeres, it would allow a cell to have two classes of telomeres, those which are long and elongated by this alternate pathway, perhaps with some contribution from telomerase, and those which are much shorter and dependent solely on telomerase. This possibility is open to experimental investigation using the newly developed peptide nucleic acid (PNA) telomere *in situ* hybridisation protocol [44], which is able to measure individual telomere length semiquantitatively. The protocol uses a $(TTAGGG)_3$ probe where the phosphodiester bonds are replaced by peptide bonds, increasing the probe's affinity for its cognate DNA sequence. This enables the length of each telomere in a single cell to be measured [44]. This analysis has now been extended to the mouse (Mark Zijlmans, Terry Fox Laboratory, Canada). $(TTAGGG)_3$ PNA detects on average the equivalent of 30–40 kb of $(TTAGGG)_n$ at each telomere in several *M. musculus* strains. This is in agreement with previous estimates of the amount of $(TTAGGG)_n$ in the mouse genome and the observation that essentially all mouse $(TTAGGG)_n$ is terminally located. Interestingly, all the mice studied had at least one telomere less than 15 kb in size, possibly equivalent to the smallest of the size distribution seen by PFGE [3, 4]. As highlighted above, an important criterion of mouse telomere structure with respect to telomere loss and cell senescence is not the average or maximum telomere size, but rather the size of the smallest telomere. In a simplistic model with all telomeres shortening at the same linear rate, the shortest telomere is likely to be the first to become 'critically short' and thus produce the hypothetical signal to cause cell cycle arrest. Both the initial PFGE data [3, 4] and the latest PNA quantitative analysis are consistent with mouse cells containing at least one telomere in the <15 kb size range. Such a telomere is within the size range of human somatic telomeres. The possibility that some mouse telomeres might be quite short has important implications for using the mouse as a model system and demands further investigation.

CONCLUSION: THE TELOMERASE KNOCK OUT MOUSE

One approach to address critically the role of telomerase in cell immortalisation and senescence is to inactivate the enzyme, either by genetic mutation or using an inhibitor. With the cloning of the RNA component of mouse telomerase it is now possible to delete this functionally essential RNA component by targeted gene knockout. What might be the phenotype of such a transgenic mouse?

It is not immediately obvious from the current data that telomeres and telomerase are critically involved in replicative senescence and immortalisation in the mouse. While the human data are internally consistent, the situation in the mouse is less clear. Even with the latest estimates of the length of the shortest telomere in a mouse cell, the presence of telomerase in many proliferating mouse somatic cells and the rate of telomere shortening in telomerase-negative mouse cells are not easy to reconcile with the much reduced proliferative capacity of mouse cells in culture compared to those from humans. Such cells, even from early embryos, typically have a life span of less than 20 population doublings, which at best would be associated with a rather modest telomere shortening of 1.5 kb.

The most extreme hypothesis is that telomeres are not involved in cell immortalisation and senescence in the mouse. As senescence *does* occur this would then have to result from another process, such as accumulation of random genome damage. As well as reconciling the long telomeres of mice with the short *in vitro* life span, this hypothesis would also permit a wide range of telomere lengths in mouse populations without any obvious phenotype (such as the two *M. spretus* populations). That mice lack a telomere-mediated block to cell immortalisation is also suggested by the relative ease with which mouse cells immortalise in culture. Such a hypothesis would still be consistent with telomeres being involved in human cell immortalisation, as this would be an additional layer of control evolved in humans against life-threatening cancer in our longer-lived species. The mouse, being short-lived, can cope with a relatively high cancer incidence rate, and *a priori* there seems much less selective pressure to evolve a telomere-mediated tumour suppression mechanism in this species.

What would be the phenotype of the telomerase knockout mouse if this hypothesis is true? Firstly, the nullizygous mouse would be viable, as stem cells would start life with sufficient telomere sequence to undergo a very large number of cell divisions before removing all the $(TTAGGG)_n$. The mouse would also be expected to show a pattern of tumour incidence and cell immortalisation in culture similar to normal mice. These phenotypes might, however, change in subsequent generations. The lack of telomerase in the germ line would mean that any telomeres whose length was dependent upon telomerase activity would shorten with each generation. In time mice might be born with at least one telomere so short that its shortening during somatic cell proliferation now had an effect, and at this point a phenotype would be seen. Whether the cell immortalisation phenotype or stem cell proliferation would be affected first is difficult to predict.

This hypothesis must also explain the data indicating an upregulation of telomerase in mouse tumours, an increase greater than that expected based on proliferative index. As suggested above, this might reflect not a requirement for telomerase in the tumours, but rather the global changes in gene expression and cell physiology that occur in tumour cells. The regulatory network controlling the processes necessary to enable a cell to be immortal may influence proteins both central and peripheral to the immortal phenotype. One natural cell type where true immortality must occur is the germ line, and here telomerase must be expressed for long-term survival of the species. Thus, telomerase upregulation is expected to be part of the co-ordinated changes between mortal and immortal cells. 'Hard-wiring' telomerase regulation into this germ:soma control mechanism could make it almost impossible for a somatic cell to become immortal without also upregulating telomerase, even though it may not be required in the medium term for cell proliferation. The possibility that telomerase upregulation in mouse tumours reflects the design of the regulatory network as opposed to selective pressure for telomerase expression in tumours can be addressed by producing a transgenic mouse lacking telomerase.

1. Kipling D. Telomerase: immortality enzyme or oncogene? *Nature Genet* 1995, **9**, 104–106.
2. Kipling D. *The Telomere.* Oxford, Oxford University Press, 1995.
3. Kipling D, Cooke HJ. Hypervariable ultra-long telomeres in mice. *Nature* 1990, **347**, 400–402.
4. Starling JA, Maule J, Hastie ND, Allshire RC. Extensive telomere repeat arrays in mouse are hypervariable. *Nucleic Acids Res* 1990, **18**, 6881–6888.
5. Kipling D, Cooke HJ. Beginning or end? Telomere structure, genetics and biology. *Hum Mol Genet* 1992, **1**, 3–6.
6. Barnett MA, Buckle VJ, Evans EP, *et al.* Telomere directed fragmentation of mammalian chromosomes. *Nucleic Acids Res* 1993, **21**, 27–36.
7. Greider CW. Telomere length regulation. *Ann Rev Biochem* 1996, **65**, 337–365.
8. Hayashi T, Mafune Y, Takada T, Hatakeyama K, Takagi N, Kominami R. Telomere change and loss of heterozygosity of mouse primary tumours and cell lines. *Jpn J Cancer Res* 1993, **84**, 1292–1299.
9. Kipling D, Salido EC, Shapiro LJ, Cooke HJ. High frequency *de novo* alterations in the long-range genomic structure of the mouse pseudoautosomal region. *Nature Genet* 1996, **13**, 78–82.
10. Yen CH, Pazik J, Elliott RW. A polymorphic interstitial telomere array near the center of mouse Chromosome 8. *Mamm Genome* 1996, **7**, 218–221.
11. Yen CH, Matsuda Y, Chapman VM, Elliott RW. A genomic clone containing a telomere array maps near the centromere of mouse Chromosome 6. *Mamm Genome* 1995, **6**, 96–102.
12. Elliott RW, Pazik J. An intersititial telomere array near Hba on mouse Chr 11 is a candidate for the homolog of the telomere at human 16p. *Genomics* 1995, **27**, 217–218.
13. Prowse KR, Greider CW. Developmental and tissue-specific regulation of mouse telomerase and telomere length. *Proc Natl Acad Sci USA* 1995, **92**, 4818–4822.
14. Garagna S, Broccoli D, Redi CA, Searle JB, Cooke HJ, Capanna E. Robertsonian metacentrics of the house mouse lose telomeric sequences but retain some minor satellite DNA in the pericentromeric area. *Chromosoma* 1995, **103**, 685–692.
15. Broccoli D, Godley LA, Donehower LA, Varmus HE, de Lange T. Telomerase activation in mouse mammary tumours: lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation. *Mol Cell Biol* 1996, **16**, 3765–3772.
16. Kipling D, Wilson HE, Mitchell AR, Taylor BA, Cooke HJ. Mouse centromere mapping using oligonucleotide probes that detect variants of the minor satellite. *Chromosoma* 1994, **103**, 46–55.

17. Kiping D, Ackford HE, Taylor BA, Cooke HJ. Mouse minor satellite DNA genetically maps to the centromere and is physically linked to the proximal telomere. *Genomics* 1991, **11**, 235–241.
18. Broccoli D, Miller OJ, Miller DA. Isolation and characterisation of a mouse subtelomeric sequence. *Chromosoma* 1992, **101**, 442–447.
19. Kipling D, Wilson HE, Thomson EJ, Cooke HJ. YAC cloning *Mus musculus* telomeric DNA: physical, genetic, *in situ* and STS markers for the distal telomere of chromosome 10. *Hum Mol Genet* 1995, **4**, 1007–1014.
20. Hayashi T, Ohtsuka H, Kuwabara K, *et al.* A variant family of mouse minor satellite located on the centromeric region of chromosome 2. *Genomics* 1993, 17, 490–492.
21. Elliott RW, Yen CH. DNA variants with telomere probe enable genetic mapping of ends of mouse chromosomes. *Mamm Genome* 1991, **1**, 118–122.
22. Eicher EM, Lee BK, Washburn LL, Hale DW, King TR. Telomere-related markers for the pseudoautosomal region of the mouse genome. *Proc Natl Acad Sci USA* 1992, **89**, 2160–2164.
23. Cross SH, Allshire RC, McKay SJ, McGill NI, Cooke HJ. Cloning of human telomeres by complementation in yeast. *Nature* 1989, **338**, 771–774.
24. Kipling D, Wilson HE, Thomson EJ, *et al.* Structural variation of the pseudoautosomal region between and within inbred mouse strains. *Proc Natl Acad Sci USA* 1996, **93**, 171–175.
25. Matsuda Y, Chapman VM. Application of fluorescence *in situ* hybridisation in genome analysis of the mouse. *Electrophoresis* 1995, **16**, 261–272.
26. Rounds D, Brueckner M, Ward DC. Isolation of murine telomere-proximal sequences by affinity capture and PCR. *Genomics* 1995, **29**, 616–622.
27. Broccoli D, Chong L, Oelmann S, *et al.* Comparison of the human and mouse genes encoding the telomeric protein, TRF1: chromosomal localization, expression, and conserved protein domains. *Hum Mol Genet* 1997, **6**, 69–79.
28. Prowse KR, Avilion AA, Greider CW. Identification of a nonprocessive telomerase activity from mouse cells. *Proc Natl Acad Sci USA* 1993, **90**, 1493–1497.
29. Blasco MA, Funk W, Villeponteau B, Greider CW. Functional characterisation and developmental regulation of mouse telomerase RNA. *Science* 1995, **269**, 1267–1270.
30. Greider CW. Telomerase biochemistry and regulation. In Blackburn EH, Greider CW, eds. *Telomeres.* New York, Cold Spring Harbor Laboratory Press, 1995, 35–68.
31. Chadeneau C, Siegel P, Harley CB, Muller WJ, Bacchetti S. Telomerase activity in normal and malignant murine tissues. *Oncogene* 1995, **11**, 893–898.
32. Holt SE, Wright WE, Shay JE. Regulation of telomerase activity in immortal cell lines. *Mol Cell Biol* 1996, **16**, 2932–2939.
33. Buchkovich KJ, Greider CW. Telomerase regulation during entry into the cell cycle in normal human T cells. *Mol Biol Cell* 1996, 7, 1443–1454.
34. Sharma HW, Sokoloski JA, Perez JR, *et al.* Differentiation of immortal cells inhibits telomerase activity. *Proc Natl Acad Sci USA* 1995, **92**, 12343–12346.
35. Blasco MA, Rizen M, Greider CW, Hanahan D. Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. *Nature Genet* 1996, **12**, 200–204.
36. Bednarek A, Budunova I, Slaga TJ, Aldaz CM. Increased telomerase activity in mouse skin premalignant progression. *Cancer Res* 1995, **55**, 4566–4569.
37. Martin GM, Austad SN, Johnson TE. Genetic analysis of ageing: role of oxidative damage and environmental stresses. *Nature Genet* 1996, **13**, 25–34.
38. Holliday R. *Understanding Ageing.* Cambridge, Cambridge University Press, 1995.
39. Campisi J. Replicative senescence: an old lives' tale? *Cell* 1996, **84**, 497–500.
40. de Lange T. Activation of telomerase in a human tumor. *Proc Natl Acad Sci USA* 1994, **91**, 2882–2885.
41. Kuroki T, Huh N. Why are human cells resistant to malignant cell transformation *in vitro*? *Jpn J Cancer Res* 1993, **84**, 1091–1100.
42. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* 1995, **14**, 4240–4248.
43. Zakian VA. *Saccharomyces* telomeres: function, structure and replication. In Blackburn EH, Greider CW, eds. *Telomeres.* New York, Cold Spring Harbor Laboratory Press, 1995, 107–137.
44. Lansdorp PM, Verwoerd NP, van de Rijke FM, *et al.* Heterogeneity in telomere length of human chromosomes. *Hum Mol Genet* 1996, **5**, 685–691.

Acknowledgements—Mark Zijlmans is thanked for permission to describe unpublished data. I am grateful to Dominique Broccoli, Maarten Linskens, Karen Prowse, Silvia Bacchetti, Sarah Bowen, and colleagues at the MRC Human Genetics Unit for their comments on the manuscript. D.K. is a Fellow of the Lister Institute of Preventive Medicine.