

# Early growth determines longevity in male rats and may be related to telomere shortening in the kidney

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**Abstract** Maternal protein undernutrition can influence the growth and longevity of male offspring in the rat. We tested the hypothesis that these differences in longevity were associated with changes in the rate of telomere shortening. We found age-related shortening of telomeres in the liver and kidney but not in the brain of male rats. Growth retardation in postnatal life was associated with significantly longer kidney telomeres and an increased longevity. Conversely, growth retardation during the foetal life followed by postnatal catch-up growth was associated with a shorter life span and shorter kidney telomeres. These findings may provide a mechanistic basis for epidemiological studies linking early growth retardation to adult degenerative diseases.

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**Key words:** Telomere; Longevity; Rat; Low protein; Kidney; Growth

## 1. Introduction

The longevity of male rats was previously shown to be increased by postnatal growth retardation but reduced by foetal growth retardation [1]. We hypothesised that the differences in longevity that we observed were due to changes in the rate of age-related telomere shortening. Telomeres are specialised nucleoprotein complexes that form the ends of eukaryotic chromosomes and in vertebrates they consist of short repeats of the sequence TTAGGG (reviewed in [2]). Shortening of human telomeres occurs during ageing [3] or when cells senesce in a tissue culture [2]. Such shortening may be an important component of ageing [4] although there are little *in vivo* data available to substantiate this [5].

Previous studies have shown that men at a mean age of 64 who at birth weighed less than 2.51 kg were 18 times more likely to exhibit a combination of hypertension, glucose intolerance and hypertriglyceridaemia than those of a birthweight over 4.31 kg [6]. This combination carries an increased risk of death from ischaemic heart disease [7]. It has been suggested that poor foetal nutrition may be a factor underlying this statistical relationship and that poor protein nutrition may be a component of this [8]. To test this hypothesis we have been studying a rat model in which pregnant and/or lactating rats are fed a diet reduced in its protein content to just under half normal. All offspring are weaned onto a normal diet fed *ad libitum*. The details of the diet, growth patterns and organ weights of the offspring of such rat dams have been published elsewhere [9]. We have measured the length of telomeres from a range of ages between 3–33 days and 13 months in the

livers, kidneys and brains of control male rats. We have also examined telomere lengths in livers, kidneys and brains of 13 months old male rats, growth retarded during foetal life (recuperated group) or suckling (postnatal low protein group).

## 2. Materials and methods

### 2.1. Animals

All procedures involving animals were conducted under the British Animals (Scientific Procedures) Act (1986). Virgin female Wistar rats with initial weights of 240–260 g were maintained individually at 22°C on a controlled 12 h light-dark cycle. They were assumed to be pregnant when a vaginal plug was observed. Rat dams were fed either a 'control' 20% protein diet or an isocaloric 'low protein' 8% protein diet (both from Hope Farms, Woerden, The Netherlands). All pups were weaned onto a 20% protein diet LAD1 (Special Diet Services, Witham, Essex, UK).

The control group was exposed to the control diet during the foetal period and lactation. Litter sizes from the control group were culled to eight pups at 2 days of age. Cross-fostering techniques were employed for the recuperated and postnatal low protein groups, using litters born on the same day. The recuperated group was offspring of mothers which were fed the low protein diet during pregnancy. Their litter size was culled to four (to maximise the postnatal exposure to good lactation) at 2 days of age after which the pups were nursed by a lactating dam fed the control diet. The postnatal low protein group was offspring of mothers which were fed a control diet during pregnancy. Their litter size was not culled (to maximise the postnatal exposure to poor lactation) and at 2 days of age, the pups were nursed by a lactating dam fed the low protein diet. The mean litter size of this group was 14.

### 2.2. Telomere DNA analysis

Genomic DNA was isolated using a modified commercial method (Puregene). 2 µg of DNA was digested overnight at 37°C with the restriction enzymes *HinfI* and *RsaI* (Boehringer Mannheim) [10] which will not cut within the (TTAGGG)<sub>n</sub> telomere repeat [3]. The digested DNA was separated by pulsed field gel electrophoresis (PFGE) (CHEF-DR II, Bio-Rad) on a 1% agarose gel (Sigma). Electrophoresis was in 0.5×TBE (0.045 M Tris base, 0.045 M boric acid, 0.001 M EDTA) at 14°C with a voltage gradient of 6 V/cm and a 1–30 s switching interval for 7 h. The gel was transferred to Hybond-N (0.45 Micron, Amersham) using 20×SSC as the transfer buffer [11] and cross-linked to the nylon using a UV Stratalinker (TM 2400, Stratagene). The DNA controls were similarly cross-linked to the control blot. The telomeric probe used was 5'-(TTAGGG)<sub>4</sub>-3' (Genosys) and was end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham) using the 5' DNA terminus labelling system (Gibco BRL Life Technologies). The radio-labelled probe was recovered using the NucTrap Probe Purification Columns (Stratagene) and analysed with the Liquid Scintillation Analyzer (1500 Tri-Carb, Packard). The blots were hybridised to <sup>32</sup>P-(TTAGGG)<sub>4</sub> using ExpressHyb (Clontech) at 50°C in a Techne Hybridiser HB-1D hybridisation oven for 1 h and then washed for 1 h at 40°C according to the manufacturers recommendations. The DNA blots were analysed using Fuji Imaging Plates (Type Bas III) and a phosphorimager FUJIX Bas 2000 machine.

### 2.3. Telomere length quantification analysis

Each DNA sample was divided into regions according to the kb size markers and the intensities (PSL) of the regions were quantified with a

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phosphorimager FUJIX Bas 2000 machine. Following the subtraction of the background (BG PSL) from each section, a percentage intensity (% PSL) for each kb size region of the total lane intensity was obtained (i.e. % PSL = PSL – BG PSL / total lane PSL – BG PSL). For each sample, the percentage intensities for the regions 145.5–48.5 kb, 48.5–9.42 kb, 9.42–4.36 kb and 4.36–1.35 kb were measured. The data were analysed using the unpaired Students' *t*-test. A standard sample, included in each gel run, was used as an exclusion mechanism to reject gels which were more than 1.5 S.D. away from the mean of standard samples run on previous gels.

#### 2.4. DNA degradation analysis

Every sample was analysed a minimum of two times to ensure that the results obtained were reproducible. DNA samples were tested for degradation by digesting 2 µg of DNA overnight at 37°C with the restriction enzyme *Eco*R1 (Boehringer Mannheim). The DNA was separated by PFGE and transferred to Hybond-N [11]. The rat satellite tandem 66 bp repeat, Ratansata [12] (Genosys), was end-labelled with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham). The blots were hybridised to <sup>32</sup>P-labelled Ratansata using ExpressHyb (Clontech) at 40°C for 1 h and washed at 37°C for 1 h. All the DNA samples showed a similar binding pattern indicating that the DNA was not degraded (results not shown).

### 3. Results

Similar to previous studies [9], the recuperated group exhibited catch-up growth during the lactation with body weights similar to controls from 21 days of age (Fig. 1A). The postnatal low protein animals however, were significantly

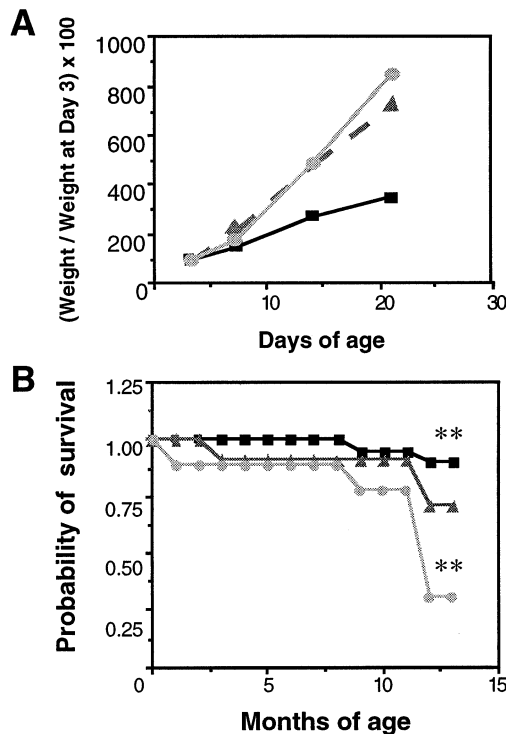


Fig. 1. Early growth and survival rates of postnatal low protein, control and recuperated males. (A) Early mean rates of growth of postnatal low protein (black square,  $n=20$ ), control (grey triangle,  $n=16$ ) and recuperated (grey dot,  $n=18$ ) males up to 3 weeks of age. (B) Survival Curves for postnatal low protein (black square,  $n=10$ ), control (grey triangle,  $n=12$ ) and recuperated (grey dot,  $n=10$ ) male rats up to 13 months of age using the Kaplan-Meier product limit method of analysis and the Wilcoxon rank-sum test (\*\* $P < 0.01$  compared to the control group).

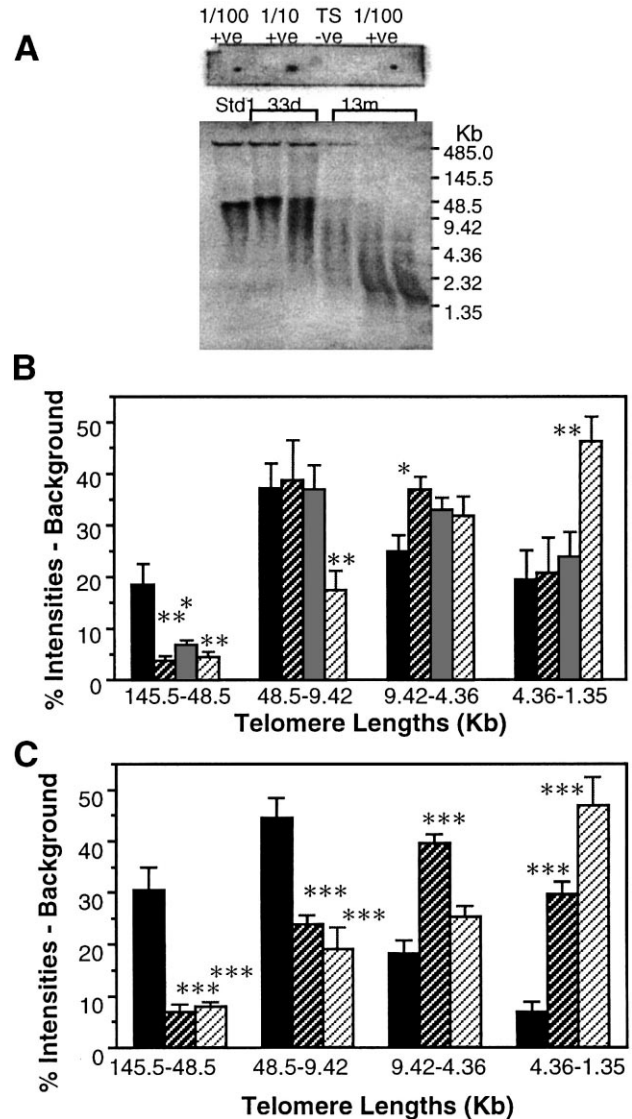


Fig. 2. Changes in liver and kidney telomere lengths with age. (A) Liver telomeres in 3–33 days and 13 months old male controls. Each lane represents a digested DNA sample which has been hybridised with the telomeric probe <sup>32</sup>P-(TTAGGG)<sub>4</sub>. Size markers (kb) derived from the Low Range PFG Marker (New England Biolabs) and <sup>32</sup>P-X174 RF DNA/Hae III Fragments (Gibco BRL) and a digested, frozen aliquoted standard sample (Std 1), used in every gel examined, are indicated. The control blot (Hybond-N nylon, 0.45 Micron, Amersham) contains three positive control dots of two 1:100 dilutions and a 1:10 dilution of (CCCTAA)<sub>3</sub> (Genosys) and one negative control dot of random DNA (TS, Genosys) which will not bind to the <sup>32</sup>P-(TTAGGG)<sub>4</sub> probe. (B) Male control liver telomeres at 3–33 days (■,  $n=5$ ), 3 months (dark hatched,  $n=8$ ), 8 months (grey,  $n=6$ ) and 13 months (light hatched,  $n=8$ ) of age. Data are means  $\pm$  S.E.M., \* $P < 0.05$ , \*\* $P < 0.01$  compared to the 3–33 day old group using the unpaired Students *t*-test. (C) Male control kidney telomeres at 3–33 days (■,  $n=8$ ), 3 months (dark hatched,  $n=6$ ) and 13 months (light hatched,  $n=8$ ) of age. Data are means  $\pm$  S.E.M., \*\*\* $P < 0.001$  compared to the 3–33 day old group using the unpaired Students *t*-test.

lighter throughout life. Survival curves of the male animals during the study period of up to 13 months of age are shown in Fig. 1B. The postnatal low protein males had a significantly higher survival rate than the controls ( $P < 0.01$ ). In contrast, the recuperated males had a lower survival rate than the con-

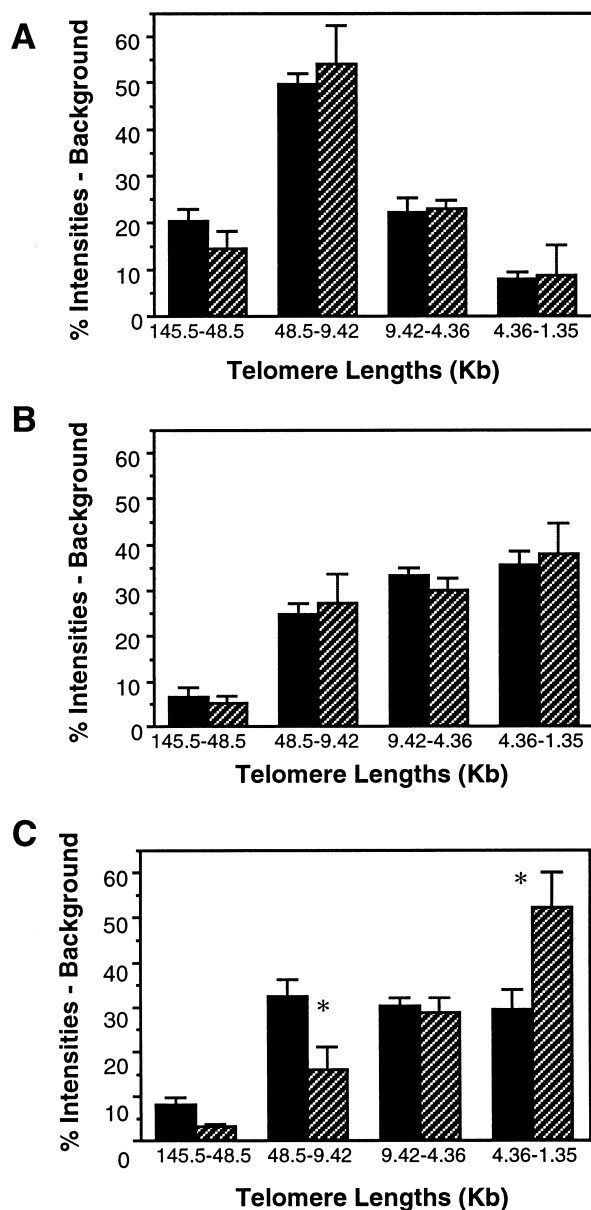


Fig. 3. Examination of telomere lengths in 13 months old postnatal low protein (■,  $n=9$ ) and recuperated (hatched,  $n=5$ ) males. (A) Brain telomere lengths, (B) liver telomere lengths and (C) kidney telomere lengths. Data are means  $\pm$  S.E.M., \* $P < 0.05$  using the unpaired Students *t*-test.

controls ( $P < 0.01$ ). In the present experiments, the device of manipulating litter sizes (see Section 2) in comparison with our previous studies allowed us to observe significant changes in longevity in quite small groups of animals.

There is increasing evidence to suggest that regardless of the mean telomere length, one critical short telomere may be the cause of a cell to enter senescence [13,14]. We therefore developed a method of analysis of telomere length which did not focus on the mean telomere length [3,15] but determined the percentage of telomeres in four different regions of length as defined by size markers [14,15]. Age-dependent shortening of telomere lengths was observed in livers and kidneys of control animals. In the liver, the 3–33 days old range of pups had significantly more telomeres in the 145.5–48.5 kb size regions compared to all the other age groups (Fig. 2A and B). The 13

months old group had the smallest telomeres, predominantly 9.42–1.35 kb in size and had significantly more telomeres than the 3–33 days old group in the 4.36–1.35 kb region. The kidney data showed a significant decrease of telomeres, 145.5–9.42 kb in size, in the 3 and 13 months old control groups and a significant increases in the 9.42–1.35 kb regions compared to the 3–33 days old control group (Fig. 2C). The reduction which we have observed in the length of liver and kidney telomeres of control male rats at 13 months is consistent with the length of telomeres becoming critical for the survival of cells during the normal life span of these animals. There is a suggestion in our liver data of three phases of telomere length change between 33 days and 13 months of age. There was initially a period of rapid shortening up to the age of 3 months with a significant depletion of the longest telomeres and an increase of those of intermediate length. Presumably, this represents the consequence of rapid growth and cell division over this period. Telomere lengths then remained fairly stable between 3 and 8 months to be followed by another pronounced phase of shortening up to 13 months. The explanation of the latter is not clear.

Liver, kidney and brain telomeres were compared in the postnatal low protein and recuperated groups at 13 months of age as it was between these groups that the greatest difference in longevity was detected. No significant difference was observed in the brain (Fig. 3A) or liver (Fig. 3B), however, significant differences were observed in kidney telomere lengths in the two groups (Fig. 3C). The recuperated group had less kidney telomeres in the 145.5–48.5 kb region ( $P = 0.057$ ) and the 48.5–9.42 kb region ( $P < 0.05$ ) and more telomeres in the 4.36–1.35 kb region ( $P < 0.05$ ) than the postnatal low protein group. The changes observed in the measurements in the recuperated group are likely to be an underestimate as the greater loss through premature death and therefore presumably the selective loss of animals with the shortest telomeres in this group may well have reduced the shortening of telomeres observed. The pattern of the brain telomere length distribution (Fig. 3A) in the 13 months old animals resembled that of the liver and kidney of young animals (Fig. 2B and C).

#### 4. Discussion

It has been known for many years that a reduction in food intake prolongs the life span of many animals including rodents [16,17]. Chronic nephropathy features strongly as a cause of death of male rats and can be strikingly reduced by dietary restriction [18]. It is therefore likely to be significant in relation to longevity that the greatest effect of early growth patterns on the telomere length was observed in the kidneys of 13 months old animals compared with the livers and brains.

The liver and kidney are expanding cell populations during postnatal life whilst, in contrast, the brain is a static cell population in which cell division ceases early in life [19]. No significant decrease of telomere length was observed in adult brain tissue as a function of age in humans [20]. The two expanding cell populations show different patterns with respect to the cell addition [21]. In the mouse, the kidney shows a continuous growth by rapid cell division initially up to 90 days of age and then slowly but continuously throughout the life span [19]. The liver grows by cell division in early life but this ceases later in life and is replaced with an increase in cell

size with age and is related to an increasing polyploidisation which occurs in the course of ageing [21]. Each doubling of the nuclear mass in polyploid cell formation results in a doubling of the nuclear and cytoplasmic volume, producing larger and larger cells (reviewed in [21]). It is not known what the consequence of telomere shortening would be in polyploid cells. Protein restriction reduces the cell size in liver and kidney [21]. As DNA replication and growth in cell size are coordinated [22], a possible mechanism for the restrained division could be a consequence of the smaller cell volumes for liver and kidney cells of rodents on postnatal protein-restricted diets [21] i.e. the postnatal low protein group. Thus, such diets may reduce the rate of cell division, telomere shortening, cell death, prolong organ function and hence increase longevity. In contrast, in our experiments, the dietary restriction was entirely maternal and restricted to short periods of 3 weeks in the early life. Nevertheless, such a dietary induced growth reduction was still associated with longevity changes. We speculate that in certain critical organs, such as the kidney, the number of cells with which the organ is endowed at birth may determine the number of cell divisions required to fulfil the organ's function in the adult life. Preservation of the organ function with ageing would depend upon the early cell mass plus the subsequent demand placed upon the organ. Others have found that intra-uterine growth retardation reduces the number of nephrons in the rat offspring at birth and this loss is unable to be compensated for during the catch-up growth [23]. Brenner has drawn attention to the potential importance of the nephron endowment at birth in relation to the risk of developing hypertension [24].

The apparent rate of telomere shortening (from a mean length of 15.34 kb at 3–33 days to a mean length of 4.97 kb at 13 months) may appear greater than that which can be explained solely by the effects of cell division. The loss of approximately 10 000 bp over a year if it occurs at the rate of 100 bp per cell division [14] would indicate that cells were dividing every 3 days. Studies of telomere shortening with cell division and with age in humans, on the other hand, might be interpreted to indicate figures of approximately a 40 bp loss per division [2] and one division per annum [3]. These figures are probably not appropriate for comparison with our data in the rat since the human longevity is so different and the effects of ageing in the human were studied in blood cells. On the other hand, rodent findings are reasonably consistent with our data. Prowse and Greider [15] measured age-related telomere shortening in *M. spretus*, a mouse with telomere lengths similar to those we found in Wistar rats. Adult mice (2–9 months) had kidney telomere lengths which were 2–3 kb shorter than those in the testis while the telomeres of these and other tissues were reported to be similar in size in new-born mice. If we assume that the mean age of these animals was 4 months and that shortening is linear with age, then these figures are consistent with our finding of 10 kb shortening over 12 months.

There is accumulating evidence that telomere shortening can occur independently of cell division. It has been suggested that telomere shortening can occur by an S-phase-specific exonuclease activity [25,26]. In addition, it has been postulated that oxidative stress can accelerate the telomere loss and hence ageing, again independently of the rate of cell division. The mechanistic basis of this telomere loss is not clear but is thought to involve free radical-mediated damage and may

be an important initiator of the cell senescence [27]. Von Zglinicki and colleagues demonstrated that an increased oxygen tension accelerated telomere shortening in replicating fibroblasts in vitro from 90 bp per population doubling under normoxia to more than 500 bp under hyperoxia and that an accumulation of telomeric DNA single strand breaks under conditions of hyperoxia was found to be dependent on the applied oxidative stress [27]. Another study demonstrated independently that age-dependent telomere shortening was decreased by the suppression of intracellular oxidative stress [28].

In a wider context, these results raise some important questions. Firstly, it is remarkable that growth restriction for a short time as the 3 weeks of lactation results in a permanent reduction in size and an increase in longevity. It will be important to determine what features of lactation are responsible and whether the increase in longevity can be dissociated from the growth retardation. Secondly, it appears that in the rat, catch-up growth is detrimental to long term survival. It is not known whether this effect is limited to this species and the particular stage in development in which it is achieved. In epidemiological studies, it has been observed that males who were born small but grew to above average height and became obese were very hypertensive [29]. Aviv and Aviv have reviewed the reasons for thinking that somatic overgrowth relative to that of the kidney might be linked to hypertension and have speculated that the telomere length is short in a subset of cell populations in the kidneys of subjects with a low birthweight [30]. Children who have suffered intrauterine growth retardation have been stimulated to grow by the administration of bio-engineered human growth hormone (reviewed in [31]). In the light of the above considerations, it would seem important to monitor the long term renal and vascular consequences of this treatment. Epidemiological studies have linked deaths from later ischaemic heart disease [32], hypertension [33] and the loss of glucose tolerance [6,34] with a reduced foetal growth. It has been observed that in endothelial cells obtained at autopsy from various human arterial sites, the age related decrease in the telomere length was the greatest at sites of higher haemodynamic stress [35]. It was speculated that focal replicative senescence might play a role in the atherogenesis. It will be of interest to determine the effects of early changes in growth on the senescence of endothelial cells. Additional key cells in which accelerated replicative senescence could be involved in the links between adult degenerative diseases and early human growth retardation, listed earlier, are the  $\beta$  cells of the islets of Langerhans. We conclude that these data are consistent with there being a mechanistic link between changes in longevity and the rate of shortening of telomeres in the kidneys of male rats. Further studies are required to determine whether genetic [36] or dietary [16,17] manipulations of the telomere length or longevity respectively provide further support for the underlying mechanism which we propose.

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