# Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity

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Loss of telomeric DNA during cell proliferation may play a role in ageing and cancer. Since telomeres permit complete replication of eukaryotic chromosomes and protect their ends from recombination, we have measured telomere length, telomerase activity and chromosome rearrangements in human cells before and after transformation with SV40 or Ad5. In all mortal populations, telomeres shortened by $\approx 65$ bp/generation during the lifespan of the cultures. When transformed cells reached crisis, the length of the telomeric TTAGGG repeats was only $\approx 1.5$ kbp and many dicentric chromosomes were observed. In immortal cells, telomere length and frequency of dicentric chromosomes stabilized after crisis. Telomerase activity was not detectable in control or extended lifespan populations but was present in immortal populations. These results suggest that chromosomes with short $(TTAGGG)_n$ tracts are recombinogenic, critically shortened telomeres may be incompatible with cell proliferation and stabilization of telomere length by telomerase may be required for immortalization.

Key words: chromosome rearrangements/immortality/telomerase/telomeres

## Introduction

Human cells can undergo only a limited number of divisions in vitro. This phenomenon, termed replicative senescence, has often been used as a model for cellular ageing (Hayflick and Moorhead, 1961; reviewed in Goldstein, 1990). Cell senescence can be at least partially overcome by carcinogen- or virus-induced transformation, a complex process that results in the acquisition of altered morphology and growth properties. Most transformed human cells acquire an extended lifespan compared with their untransformed counterparts, but ultimately cease to divide and die (crisis). At a very low frequency, some cells overcome both replicative senescence and crisis and become immortal (reviewed in Sack, 1981; DiPaolo, 1983; Chang, 1986).

As somatic cells age in vivo or in vitro, their telomeres become progressively shorter (Harley et al., 1990; Hastie et al., 1990). Telomeres are specialized structures at the ends

of eukaryotic chromosomes, consisting of proteins and simple repeated DNA sequences which are highly conserved throughout evolution (Blackburn and Szostak, 1984; Moyzis et al., 1988; Meyne et al., 1989; Zakian, 1989). In humans, 5–15 kbp of TTAGGG repeats are found at the ends of all chromosomes (Allshire et al., 1988; Moyzis et al., 1988; Brown, 1989; Cross et al., 1989; de Lange et al., 1990). Telomeres are elongated by the ribonucleoprotein enzyme telomerase which adds telomeric sequences de novo (Greider and Blackburn 1985, 1987). Their structure and mode of synthesis thus allow for the complete replication of chromosome ends. In addition, telomeres protect chromosome ends against illegitimate recombination and may direct chromosome attachment to the nuclear membrane (reviewed in Blackburn and Szostak, 1984; Blackburn, 1991; Zakian, 1989).

Loss of chromosome terminal sequences with each round of replication had previously been predicted based on the inability of DNA polymerases to completely replicate linear DNA molecules (Olovnikov 1971, 1973; Watson, 1972). Olovnikov (1973) further proposed that telomere shortening would ultimately lead to cell death and thus might play a role in cell senescence by limiting proliferation of somatic cells. Unicellular organisms and germline cells, however, should have acquired a mechanism to overcome incomplete replication of chromosome ends, thus allowing for their unlimited proliferative capacity (Olovnikov, 1973; reviewed in Harley, 1991). In agreement with these predictions, telomerase activity has been detected in unicellular eukaryotes where telomere length is stable (Greider and Blackburn, 1985, 1989; Zahler and Prescott, 1988; Shippen-Lentz and Blackburn, 1989) and cell senescence results from mutations in the Est1 (ever shorter telomeres) gene of yeast (Lundblad and Szostak, 1989) or in the RNA component of Tetrahymena telomerase (Yu et al., 1990). Moreover, in human germline cells, telomeres are significantly longer than those from somatic tissues (Allshire et al, 1989; Cooke et al., 1989; Cross et al., 1989; de Lange et al., 1990; Hastie et al., 1990) and are stable regardless of donor age (Allsopp,R.C., Vaziri,H., Patterson,C., Goldstein,S., Younglai,E.V., Futcher,A.B., Greider,C.W. and Harley,C.B., submitted). Presumably, telomerase is active in the germline, but inactive in somatic cells where telomere shortening occurs.

A role for telomere shortening in cellular senescence would further imply that immortalized cells should have telomerase activity and stable telomeres (reviewed in Harley, 1991). Enzyme activity has indeed been detected in HeLa cells, which are tumour-derived and immortal (Morin, 1989). However, loss of telomeric sequences can occur in tumour cells since often their telomeres are shorter than those in the tissue of origin (Cooke et al., 1985; de Lange et al., 1990; Hastie et al., 1990). Tumour and transformed cells, like senescent cells, also have dicentric chromosomes which may arise from telomere association and fusion as a direct

result of sequence loss (Wolman *et al.*, 1964; Benn, 1976; Blackburn and Szostak, 1984; Pathak *et al.*, 1988; Zakian, 1989).

The existence of shortened telomeres in many tumour cells suggests that telomere stabilization and expression of telomerase activity occur relatively late during oncogenesis. This might also be the case for cell transformation *in vitro*. We hypothesized that loss of telomeric sequences would not be arrested in transformed cells which have acquired only an extended lifespan and might in fact play a role in the proliferative crisis of these cells. However, activation of telomerase, and thus telomere stabilization, would necessarily occur during the development of the immortal phenotype.

To test this hypothesis we measured the length and amount of telomeric DNA, incidence of dicentric chromosomes and telomerase activity throughout the lifespan *in vitro* of human embryonic kidney cells, untransformed or transformed by SV40 or Ad5 oncogenes. Our results indicate that telomeric sequences were initially lost at a similar rate in all cell populations. This process continued unchecked in transformed populations which had acquired only an extended lifespan and died at crisis. In such populations no telomerase activity was detected and loss of telomeric DNA was associated with an increase in dicentric chromosomes. In contrast, populations which had become immortal expressed telomerase and in these cells we observed stabilization of telomeric DNA length and of the frequency of dicentric chromosomes.

## Results

### Decrease in length and amount of telomeric DNA with cell age in vitro

To define the relationships between telomeres and cell proliferation, the length and amount of telomeric DNA were measured throughout the lifespan of transformed cells generated by transfection of human embryonic kidney (HEK) cells with the plasmid pSV3neo (Stewart and Bacchetti, 1991; Bacchetti,S. and LeFeuvre,C.E., unpublished). Under G418 selection a total of 11 colonies expressing SV40 tumour antigens were isolated and expanded. All of the populations (Table I) were found to have an extended lifespan compared with the control untransfected HEK cells, which could be subcultured for only 16 mean population doublings (MPD). However, ten of the populations (HA2-EL to HA11-EL) entered a crisis between 75–120 MPD, from which viable cell lines could not be rescued. One population, HA1-IM, underwent a brief period of slow growth from which, at ≈90 MPD, evolved a faster growing subclonal population

characterized by the presence of a marker chromosome. HA1-IM cells have since continued to proliferate for >250 MPD and are therefore considered immortal. A second immortal population, the 293 cell line generated by transformation of HEK cells with adenovirus 5 DNA (Graham *et al.*, 1977), was also cultured for analysis.

Genomic DNA was isolated every two or four MPD throughout the lifespan of control cells and of a subset of the SV40 transformed cells. For the 293 cells, DNA was obtained at one time point just prior to crisis from the pre-immortal population and periodically between 12 and 135 MPD from the post-crisis immortal population. The DNA was cleaved with *Hin*fI and *Rsa*I to liberate the terminal restriction fragments (TRFs, Figure 1A) which comprise both subtelomeric repetitive DNA, X, and telomeric TTAGGG repeats, T. TRFs were resolved on agarose gels and detected by hybridization to the telomeric probe $(CCCTAA)_3$ (Figure 1B). Heterogeneity in TRF size and its multimodal distribution are due to variations in both the number of TTAGGG repeats and the length of the subtelomeric region X, but the latter accounts for most of these effects (Prowse,K.R., Abella,B.S., Futcher,A.B., Harley,C.B. and Greider,C.W., submitted). Thus, upon autoradiography the TRF specific signal appears as a smear often divided in subpopulations.

TRF length decreased with increasing MPD in all cell populations analysed (Figure 1B). This trend was most evident for the high molecular weight band (≈17 kbp), particularly in the HA1-IM DNA, but was also easily detected for all subpopulations of TRFs sizes in both HA1-IM and HA5-EL DNA. In control HEK cells, analysis of the limited number of samples which could be obtained indicated an overall reduction in TRF size, but the relatively high molecular weight of the TRFs precluded detection of subpopulations. A reduction in TRF length with increasing MPD also occurred in the 293 cells, since TRFs in the population just prior to crisis were shorter than in HEK cells but slightly longer than in the immortal population which emerged from crisis (not shown).

A decrease in the total hybridization signal, i.e. in the amount of DNA complementary to the $(CCCTAA)_3$ probe, was also apparent in all samples analysed. This suggested that shortening of TRFs is due to specific loss of the TTAGGG repeats in the T region, rather than to a decrease in the subtelomeric region X (Figure 1A). Low molecular weight bands of ≈2, 1.6 and 0.9 kbp did not decrease in size or intensity with age of the cells (Figure 1B) and were insensitive to digestion of genomic DNA with exonuclease Bal31 (not shown), which progressively removes telomeric sequences (reviewed in Blackburn and Szostak, 1984). These

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**Table I.** Growth and cytogenetic properties of cells

| Cells | Transforming viral oncogenes | Lifespan | Increase in dicentrics near crisis |
|---|---|---|---|
| HEK | | mortal, cell death at MPD 16 | – |
| HA2,3,4,5-EL | SV40 T and t | extended, crisis at MPD 75 | + |
| HA6,7,8,9,10,11-EL | SV40 T and t | extended, crisis between MPD 100 and 120 | +[a] |
| HA1-IM | SV40 T and t | immortal (passaged >250 MPD), crisis MPD 65–90 | + |
| 293 | Ad5 E1 | immortal | n.d. |

[a]HA6,8,9 and 11-EL were not tested
n.d. not determined

bands represent nontelomeric DNA fragments which hybridize at high stringency to the $(CCCTAA)_3$ probe and they were used as an internal control for the amount of DNA loaded.

The average TRF length and hybridization signal were quantified by densitometry (Figure 2 and Table II). Telomeric sequences were lost at an average rate of $\approx 65$ bp/MPD in the extended lifespan and pre-immortal populations ($\Delta$TRF/MPD in Table II). The data for HEK cells indicated a loss of $\approx 150$ bp/MPD. However, this higher loss rate may not be significant since HEK TRFs could only be measured in a limited number of samples and their large size made accurate measurements difficult. The rate at which telomeric sequences were lost in pre-crisis 293 cells was not determined.

In all SV40 transformed cell populations, TRFs decreased until crisis when their average length was $\approx 4$ kbp. In the immortal HA1-IM population, past 90 MPD, TRF length stabilized indicating no further loss of telomeric sequences for at least 120 additional MPD (Figure 2A). Loss of telomeric DNA also ceased in the second immortal line analysed, the post-crisis 293 cells, where stable TRFs of $\approx 3$ kbp average length were maintained for at least 135 MPD (Figure 2B; Table II). The 293 cells used in these experiments were from monolayer cultures. TRFs of similar

length were present in the nonadherent 293-N3S cells, whereas in the nonadherent 293(CSH) subline the TRFs had an average size of $\approx 7.5$ kbp. Since both nonadherent lines were derived from immortal adherent cells well beyond crisis, this suggests that telomere elongation might have occurred in the 293(CSH) cells, although clonal variation cannot be ruled out (see Discussion).

The amount of telomeric DNA, as quantified from the total TTAGGG hybridization signal, also decreased at a similar rate in both extended lifespan and pre-immortal populations transformed by SV40 (Figure 2B; Table II: $\Delta$S/MPD). The results for control HEK cells indicated a higher rate of decrease in signal intensity than for the transfected cells but, as noted above, quantification of these samples was less accurate. In the HA1-IM cells no further loss of TTAGGG signal was detected past MPD 90. Similarly, the amount of telomeric DNA was also stable in the immortal 293 cell line past crisis (Figure 2D).

### The mean telomeric TTAGGG DNA length at crisis is $\approx 1.5$ kbp

It has been hypothesized that loss of telomeric sequences may play a role in cell mortality (Olovnikov, 1973; Harley *et al.*, 1990; Hastie *et al.*, 1990). Since the TRF contains both subtelomeric and TTAGGG sequences, we determined
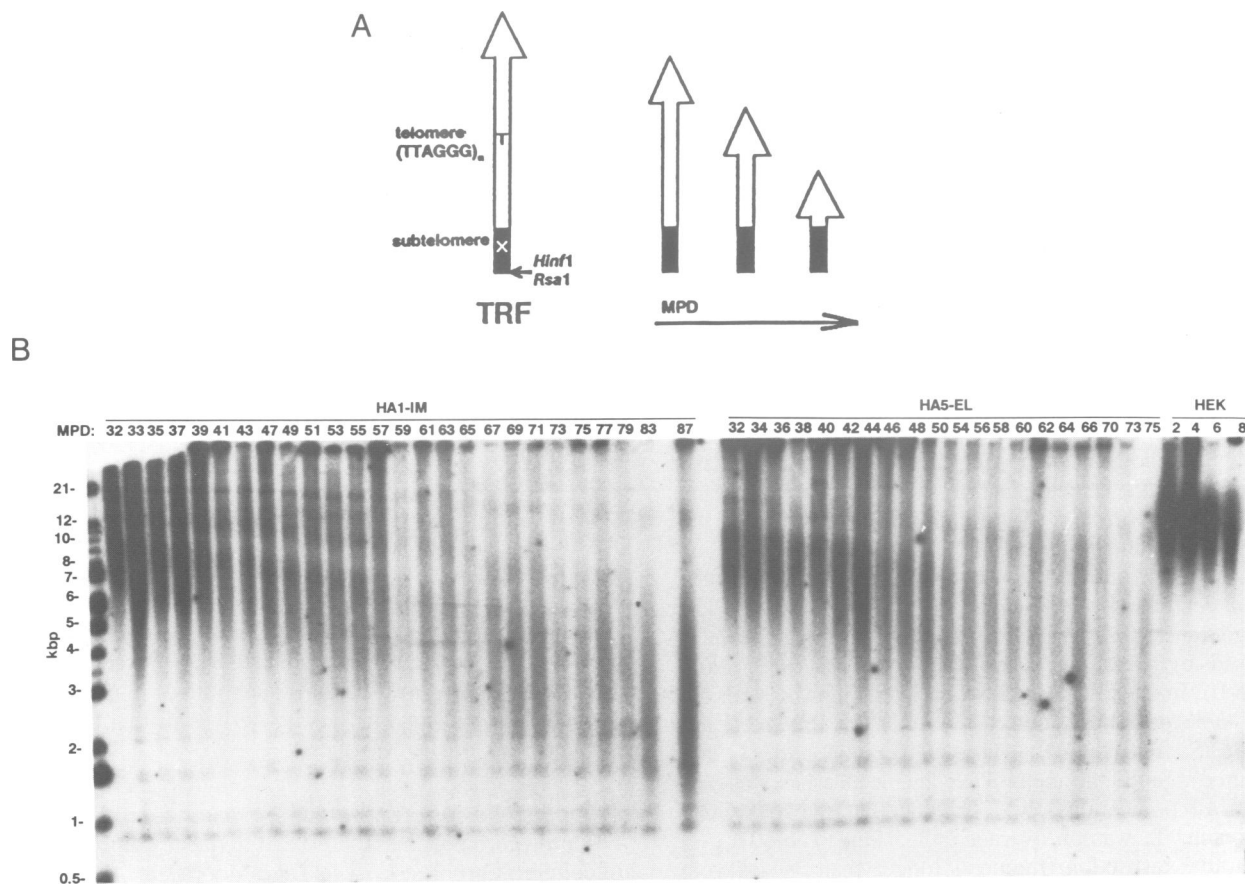


Fig. 1. (A) Structure of terminal restriction fragments (TRFs) and model for TRF shortening. TRFs are comprised of telomeric DNA, T, consisting of TTAGGG repeats and of subtelomeric DNA, X, containing degenerate arrays of TTAGGG and other sequences. As cells proliferate, TRFs become progressively shorter through loss of T. (B) Length of TRFs versus age (MPD) in immortal (HA1-IM), extended lifespan (HA5-EL) and control HEK cells. DNA was extracted from each cell population at the indicated MPD, digested with restriction enzymes *Hin*fI and *Rsa*I, separated by electrophoresis on a 0.5% agarose gel and hybridized to the human telomeric probe [32P](CCCTAA)₃. In all cases 1.0 μg of DNA was loaded, except for the HA1-IM sample at 87 MPD which was overloaded. The auroradiograph represents a typical result and is one of several experiments with these cell populations. Similar patterns were observed for all other cell populations analysed.
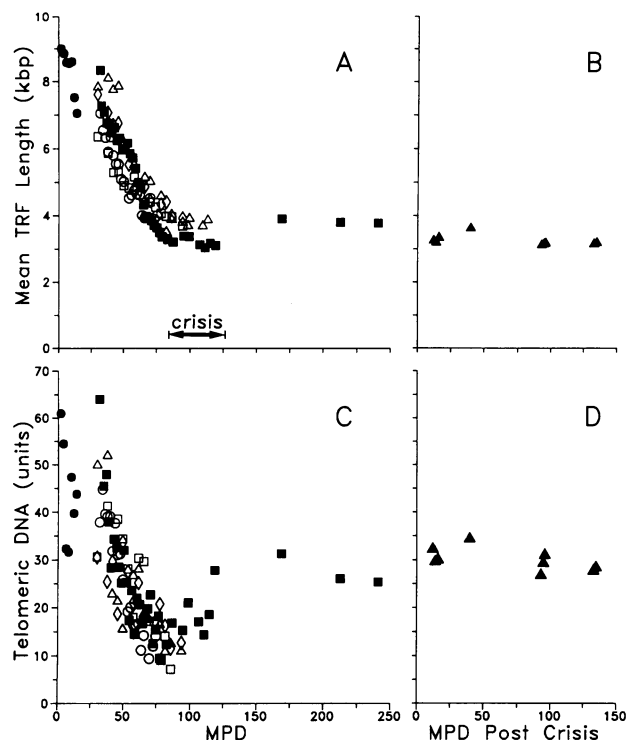
**Fig. 2.** Mean TRF length and amount of telomeric DNA as a function of age of control and transfected cells. For each data point a minimum of three autoradiographs (such as that of Figure 1B) were scanned with a densitometer over the size range 2−21 kbp and the densitometric values were used to determine the mean TRF length in kbp (**panel A** and **panel B**) and the amount of telomeric DNA (**panel C** and **panel D**). The latter is expressed in arbitrary units and was derived from the intensity of the total hybridization signal as previously described (Harley *et al.*, 1990). The average standard deviation of the data points for mean TRF length was 0.5 kbp with the largest deviation being 1.1 kbp. For the amounts of telomeric DNA the average and the largest standard deviations were 9.1 units and 28.6 units, respectively. Values generated from: ●, control HEK; ○, HA5-EL; □,HA6-EL; △, HA7-EL; ◇, HA10-EL; and ■, HA1-IM populations are plotted in panels A and C; the span of MPD at which individual populations enter crisis is indicated by the bar. Values generated from 293 cells (▲) are shown in panels B and D.

the length of the latter component in cells undergoing crisis. Assuming that the decrease in TRF length and signal intensity results solely from loss of terminal TTAGGG repeats, the length of the subtelomeric component of the TRF, X, can be calculated by comparing the rate of signal loss with the rate of decrease in TRF length (Levy *et al.*, 1992). The average subtelomere length, X, for all DNA samples we have analysed by *Hin*fI and *Rsa*I digestion was ≈2.5 kbp (Table II). In other human cell strains, the length of X ranged from ≈3 to 4 kbp (Levy *et al.*, 1992; Prowse,K.R., Abella,B.S., Futcher,A.B., Harley,C.B. and Greider,C.W., submitted). Since the mean TRF length at crisis was ≈4 kbp in all populations, we conclude that the TTAGGG component, T, was on average ≈1.5 kbp (Table II).

Terminal restriction fragments longer than 3−4 kbp at crisis are clearly seen in Figure 1B. We have analysed three subpopulations of long TRFs: the ≈17 kbp band from HA1-IM cells (Figure 1B) and the ≈15 kbp bands from HA7-EL and HA10-EL cells (not shown). We found that these TRFs decreased in length at the same rate as the mean of the total TRF population (Figure 3). Calculations such as those described above indicated that the average length
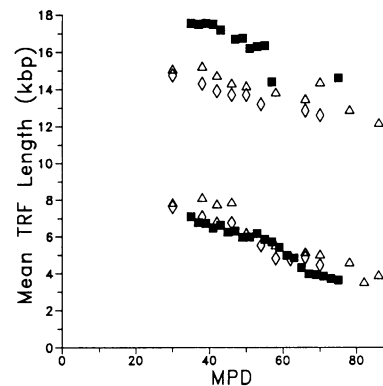


**Fig. 3.** Comparison between the length of the high molecular weight TRF subpopulation and the mean TRF length. The high molecular weight bands (see Figure 1B) in DNA isolated at the indicated MPD from HA1-IM (■), HA7-EL (△) and HA10-EL (◇) were scanned with a densitometer and their mean TRF lengths determined. These values ranging from 18 to 13 kbp are plotted together with those of the average of all TRF (ranging from 8 to 3 kbp) for the same cell populations and MPD.



**Fig. 4.** Cytogenetic analysis of SV40 transformed cells. Chromosome spreads were prepared and scored as described in Materials and methods. A metaphase with different types of aberrations is shown: dc, dicentric chromosome; dm, double minute; b, break; m, marker chromosome.

of TTAGGG DNA present in the high molecular weight TRFs at crisis was 1.5 kbp, as was that of the total TRF population (Table II). Thus long TRFs do not necessarily represent long TTAGGG tracts. In agreement with data obtained from analysis of human fibroblasts (Levy *et al.*, 1992; Prowse,K.R., Abella,B.S., Futcher,A.B., Harley,C.B. and Greider,C.W., submitted) it appears that the heterogeneity in TRF length derives primarily from a variable size of the X region.

### Chromosome fusion products increase near crisis

Dicentric chromosomes increase in frequency during ageing of cultured fibroblasts (Saksela and Moorhead, 1963; Benn, 1976; Sherwood *et al.*, 1988; reviewed in Harley, 1991) and are abundant in transformed and tumour cells (Moorhead and Saksela 1963, 1965; Wolman *et al.*, 1964; Pathak *et al.*, 1988). Since chromosomes which have lost their telomeric DNA are highly recombinogenic (Blackburn and Szostak, 1984; Pathak *et al.*, 1988; Zakian, 1989), we investigated whether the existence of very short telomeric DNA in cells

**Table II.** Characteristics of telomeres in control and transfected cells

| Cells | ΔTRF/MPD[a] (bp/MPD) | ΔS/MPD[a] (units/MPD) | TRF length at crisis (kbp) | T length at crisis (kbp) | X length (kbp) |
|---|---|---|---|---|---|
| HEK HA5-EL, HA6-EL, | −150 | −1.2 | n.d. | n.d. | 2.6 |
| HA7-EL, HA10-EL | −62 ± 17 | −0.5 ± 0.2 | 3.9 ± 0.1 | 1.5 ± 1.0 | 2.3 ± 0.9 |
| HA1-IM | −82[b] | −0.7[b] | 3.5[c] | 1.0[c] | 2.5 |
| 293 (adherent) | n.d. | n.d. | 3.2[c] | n.d. | n.d. |

[a]ΔTRF/MPD and ΔS/MPD are the decrease in TRF length and signal intensity respectively, as a function of MPD. They are derived from the slopes of the linear regressions of the data points of each cell population from Figure 3 [upper (A) and lower (C) panels respectively].
[b]Before MPD 90
[c]Average values from post-crisis cells
n.d. not determined. HEK cells do not undergo crisis, thus no values for the lengths of TRF and T at crisis are presented. Since adherent 293 cells were assayed at one time point prior to crisis and the TRF length and the amount of telomeric DNA were stable after crisis (Figure 3), no values were calculated for ΔTRF/MPD, ΔS/MPD, T and X lengths. Note also that the average values of X, T and TRF at crisis for all HA clones tested are 2.5 kbp, 1.5 kbp and 4 kbp, respectively.

at crisis was accompanied by an increase in the incidence of chromosome rearrangements which could potentially derive from telomere association and fusion. At early times after isolation all transformed populations contained on average less than one dicentric per 50 metaphases (Figure 4). Just prior to crisis, when the least amount of telomeric DNA was present, we observed a dramatic increase in the frequency of these rearrangements (Figure 5 and Table I). Ring chromosomes were also detected but at a very low frequency. Beyond crisis, in the HA1-IM cells past 90 MPD, the number of dicentrics stabilized or possibly even decreased (Figure 5). Dicentric chromosomes can arise from mechanisms other than telomere fusion and in fact other types of aberrations, unrelated to telomeres, increased prior to crisis and stabilized thereafter (not shown; Stewart and Bacchetti, 1991). Nevertheless, the striking correlation between the kinetics of telomeric DNA loss and of appearance of dicentric chromosomes, suggests the possibility that at least a fraction of these rearrangements might have arisen from telomere fusion. No chromosome aberrations were detected in 300 metaphases of control HEK cells at 10 MPD, beyond which time the low mitotic index of the cultures precluded significant cytogenetic analysis (not shown).

### Telomerase is active in immortal cells

Telomeric DNA decreased in length in all populations analysed until crisis, when the cells either died or became immortal and maintained constant telomere length. To determine if telomerase activity was correlated with telomere stability, we assayed extracts from the SV40 transformed cells and the Ad5 transformed 293 cells. Telomerase activity *in vitro* is detected as a characteristic 6 nt repeat ladder on sequencing gels (Greider and Blackburn, 1985; Morin, 1989). This pattern was visible using extracts from the immortal 293 cells (Figure 6A) and HA1-IM cells at 269 MPD (Figure 6A) or 123 and 165 MPD (Table III). In both cell lines, pre-incubation of the extracts with RNase, which inactivates telomerase, abolished formation of the 6 nt repeat ladder. The RNase-insensitive 10 nt repeat ladder seen in lanes 9 and 10 between the 75 and 142 size markers, as well as other RNase insensitive bands, were observed sporadically in reactions with extracts from monolayer cultures harvested by scraping (Materials and methods). These patterns were independent of the presence of the telomeric oligonucleotide primer and arise from *in vitro* labelling of contaminating

DNA. No telomerase activity was detected in extracts from the extended lifespan populations, HA5-EL and HA10-EL (Figure 6A), the HA1-IM population at 45 or 75 MPD, or the control HEK cells (Table III). All these cells were assayed before crisis or senescence when telomere shortening was occurring. The lack of enzyme activity in these extracts was not due to limited sensitivity of the assay since activity was detected in extracts of 293 cells diluted by as much as 80-fold (not shown), nor was it due to a diffusible inhibitor since mixing of HA5-EL and 293 cell extracts gave similar levels of activity as the 293 extract alone (Figure 6B). Lastly, telomerase negative extracts were as active as positive extracts in DNA polymerase assays (not shown) attesting that no generalized protein inactivation had occurred. Although the lack of detectable telomerase activity is not definitive proof for the absence of the enzyme, the detection of activity in two independently immortalized cell lines was quite striking. These results suggest that activation of the telomerase occurred during the establishment of the immortal populations.

### Discussion

We have documented three novel aspects of the process of cell immortalization. Firstly, we have shown that critically short telomeres are associated with an increased frequency of dicentric chromosomes and with crisis of transformed cell populations. Secondly, in immortal cells, shortening of telomeres is arrested and the frequency of dicentric chromosomes stabilizes. Lastly, we have found that telomerase activity is not detectable in populations undergoing telomere loss, but is present in immortal cells where a constant telomere length is maintained.

### Telomere shortening occurs in transformed cells which are not immortal

All transfected HEK cell populations expressing SV40 tumour antigens were found to lose telomeric TTAGGG repeats progressively till the onset of crisis. The average rate of loss of ≈65 bp/MPD, although possibly slower than that in untransfected HEK cells, was similar to that previously reported for human diploid skin fibroblasts (Harley *et al.*, 1990). Thus, acquisition of transformed properties such as altered growth rate and extended lifespan did not prevent the shortening of telomeres which is characteristic of senescing cells (Harley *et al.*, 1990) and which is probably
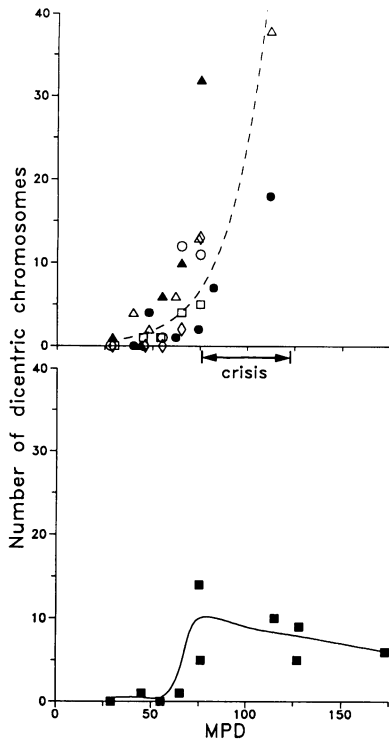
**Fig. 5.** Frequency of dicentric chromosomes as a function of age of transformed cell populations. At the indicated MPD chromosome spreads were prepared from: ●, HA2-EL; □, HA3-EL; ▲, HA4-EL; ○, HA5-EL; △, HA7-EL; ◇, HA10-EL (**upper panel**) and ■, HA1-IM (**lower panel**). For each sample, 50 metaphases were analysed for the presence of chromosome aberrations. The number of dicentric chromosomes per 50 metaphases is plotted versus the age of the populations in MPD. Similar curves were obtained when plotting the frequency of cells with dicentrics, since in the majority of cases one dicentric was present per cell. As indicated, crisis refers to the range of MPD at which individual populations enter this period.

a consequence of incomplete replication of chromosome ends (Watson, 1972; Olovnikov, 1973; Levy *et al.*, 1992).

We calculated that the average length of telomeric TTAGGG DNA was ≈ 1.5 kbp at crisis. Even long restriction fragments were found to contain short TTAGGG tracts, in agreement with the suggestion that heterogeneity in TRF length derives primarily from the variable size of the subtelomeric region (Levy *et al.*, 1992; Prowse,K.R., Abella,B.S., Futcher,A.B., Harley,C.B. and Greider,C.W., submitted). Since the value of ≈ 1.5 kbp is an average for the entire TRF population, it seems likely that at least some cells contain chromosomes with little or no protective telomeric DNA. Indeed, given the average rate of base pair loss per MPD and assuming that the TTAGGG region of the telomeres is roughly the same length in all chromosomes, we estimated that TRFs should lose all telomeric repeats by ≈ 100 MPD. Support for the generation of unprotected chromosome ends comes from the large increase in the number of dicentric chromosomes detected in cells at crisis. Association and fusion of chromosome ends occur in the absence of telomeric DNA (Blackburn, 1991; Zakian, 1989) and have been frequently observed in senescent and tumour cells and in cells transformed by SV40 (Benn, 1976; Fitzgerald and Morris, 1984; Walen, 1987; Meisner *et al.*, 1988; Pathak *et al.*, 1988 and references therein). It seems likely that this type of illegitimate recombination might have contributed to the formation of dicentrics in the transformed
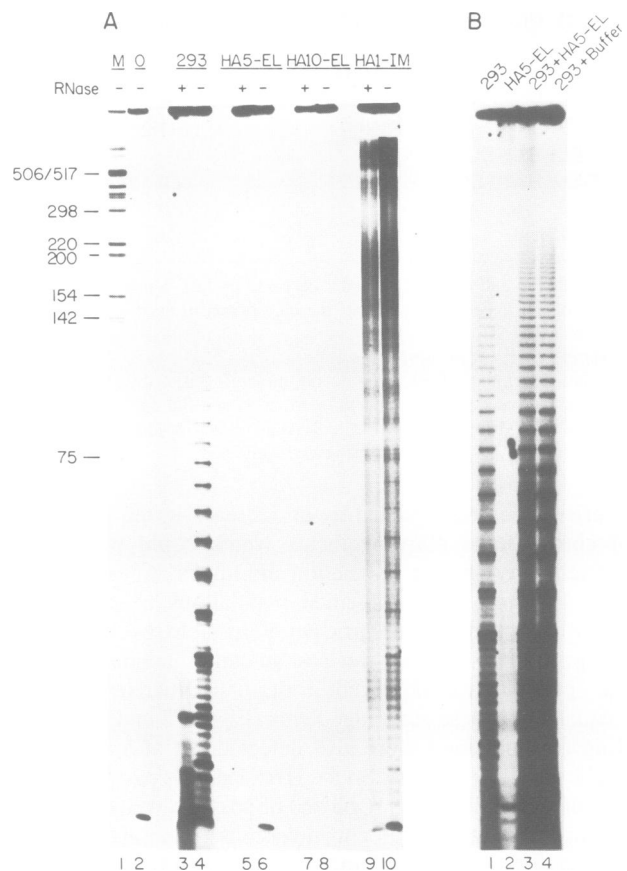


**Fig. 6.** Telomerase activity in extended lifespan and immortalized populations. (**A**) Each of the indicated cell populations was tested for telomerase activity using 80 μl reactions in the presence (lanes 4, 6, 8 and 10) or absence (lanes 3, 5, 7 and 9) of RNase. A $^{32}$P-labelled 1 kbp ladder (lane 1, M) and the $[^{32}P](TTAGGG)_3$ oligonucleotide (lane 2, O) were run as markers. 293 cells (lanes 3 and 4) and HA1-IM cells (lanes 9 and 10) are immortal. In this experiment 293(CSH) cells and HA1-IM cells at 269 MPD were assayed. Telomerase activity was also detected in the adherent 293 cells and in the 293-N3S subline (Table III). HA5-EL (lanes 5 and 6) and HA10-EL (lanes 7 and 8) are populations with extended but finite lifespan; these cells were assayed at 47 and 57 MPD, respectively. (**B**) Extracts from HA5-EL and 293 cells were mixed and assayed for telomerase activity to determine if an inhibitor was present in the HA5-EL extract. Both extracts were assayed individually (lanes 1 and 2) using 20 μl of extract in a 40 μl reaction volume. In the mixing experiment, 20 μl of HA5-EL extract was combined with 20 μl of 293 extract (lane 3) or 20 μl of buffer (lane 4) in a final volume of 80 μl.

populations we have analysed. The fact that stabilization of telomeres in the immortal HA1-IM population correlates with a decrease in the frequency of these rearrangements is in agreement with this hypothesis. Thus, crisis in SV40 transformed cells could in part result from the existence of one or more chromosomes with 'critically' short telomeres.

Untransformed HEK cells, like other cells of epithelial origin, have an unusually short lifespan in culture compared with human embryonic fibroblasts. In part, this might reflect a lack of specific growth factors in the medium (Stampfer *et al.*, 1980; Chang, 1986), as also suggested by the absence of chromosome aberrations associated with true senescence in late cultures of these cells (this study; Stewart and Bacchetti, 1991). Nevertheless, loss of telomeric sequences occurred in HEK cells and such loss (from ≈ 9 to 7 kbp) was comparable to that observed during the lifespan of

**Table III.** Correlation between telomerase activity and immortalization

|  | Cells | MPD | Telomerase activity[a] |
|---|---|---|---|
|  | HEK | ≈ 10 | – |
| pre-crisis | HA5-EL | 47 | – |
|  | HA10-EL | 57 | – |
|  | HA1-IM | 45 | – |
|  |  | 75 | – |
| post-crisis | HA1-IM | 123 | + |
|  |  | 165 | + |
|  |  | 269 | + |
|  | 293 (adherent) | 22 | + |
|  | 293 (CSH and N3S) | n.d.[b] | + |

[a]Telomerase activity in S100 extracts from cells at the indicated MPD was detected by extension of a telomeric oligonucleotide as described in Figure 6. Extracts were considered negative if the characteristic 6 nt repeat pattern was not detected on sequencing gels within a two week exposure of the film.
[b]Cumulative MPD of nonadherent 293 cells was not determined.

human embryonic fibroblasts (from ≈ 8 to 6 kbp over 60 MPD; Harley *et al.*, 1990). Transformation with viral oncogenes confers an extended lifespan to both HEK cells and fibroblasts (Girardi *et al.*, 1965; Stein, 1985; Radna *et al.*, 1989; Shay and Wright, 1989; Wright *et al.*, 1989). Thus, senescence in primary human cells may represent a checkpoint rather than an absolute limit to cell proliferation (Wright *et al.*, 1989; reviewed in Goldstein, 1990). Telomere shortening could be one of the signals that triggers this checkpoint and causes cell cycle exit and cell response or threshold levels may differ in various tissues. Viral transformation may override this signal and cause cells to resume division but, in the absence of telomerase, loss of telomeric DNA would continue until the cells reach crisis.

### Telomerase may be required for cell immortality

The most interesting observation from our analysis of telomere dynamics in transformed cells was that loss of telomeric DNA ceased in two distinct populations of immortalized cells, HA1-IM and 293. In HA1-IM cells, where telomere shortening could be measured throughout the pre-crisis growth, stabilization of telomere length occurred quite abruptly at ≈ 90 MPD. This trend most likely reflects the outgrowth of a subclonal population which had acquired the immortal phenotype including the ability to halt telomere loss. The HA1-IM population which emerged from an indistinct crisis associated with slow growth prior to 80 − 90 MPD, was characterized by a higher growth rate and the presence of a marker chromosome. The progenitor of the immortal subclone must, however, have been present in the HA1-IM population earlier since two different stocks of early passage HA1-IM cells were successfully grown past crisis and yielded immortal lines with the same marker chromosome. It is tempting to speculate on the potential role of this aberrant chromosome. It was first detected in one of 50 cells at 75 MPD but might conceivably have existed in the population even earlier (Stewart and Bacchetti, 1991). However, its occurrence at similar low frequencies in transformed cell populations which could not be rescued from crisis (e.g. HA4-EL and HA5-EL; Stewart and Bacchetti, 1991), suggests that it may represent a genetic change which is necessary but not sufficient for the acquisition of the immortal phenotype. Other events must

presumably occur to allow unlimited proliferation of cells.

We propose that expression of telomerase is one of the events required for a cell to acquire immortality. Telomerase activity was not detected in untransfected HEK cells nor in cells which expressed the SV40 tumour antigens and some of the transformed phenotypes but still retained a limited lifespan. In contrast, the enzyme was present in extracts of SV40 or Ad5 transformed cells which had become immortal and its presence was correlated with that of stable telomeres. This pattern of expression indicates that the enzyme is not induced by the viral proteins and suggests the involvement of mutational events. It is possible that one or more of the chromosomal rearrangements associated with shortened telomeres resulted in telomerase reactivation. In addition to the two types of virus transformed HEK cells reported here, telomerase activity has been detected in two human tumour cell lines, HeLa (Morin, 1989) and HL-60 (Allsopp,R.C., Vaziri,H., Patterson,C., Goldstein,S., Younglai,E.V., Futcher,A.B., Greider,C.W. and Harley,C.B., unpublished). We have previously argued that shortening of telomeres might contribute to replicative senescence both *in vivo* and *in vitro* (Harley *et al.*, 1990), a hypothesis which was at variance with the existence of immortal cells with short telomeres (de Lange *et al.*, 1990; Hastie *et al.*, 1990). Our findings that in immortalized cells telomeres are stable and telomerase activity is present resolve this apparent contradiction.

Our data allow us to define an approximate time in the growth of the immortal HA1-IM population when the enzyme is first expressed. Although extracts from cells before crisis (45 and 75 MPD) were negative for telomerase, enzyme activity could have been present but undetected in a small fraction of the population. However, the telomere length of HA1-IM cells past 90 MPD indicated that the progenitor of the immortal population acquired telomerase activity when its telomeres were critically shortened. Similar conclusions, as to the time of telomerase expression, can be drawn for the immortal 293 cell line, which also has stable but very short telomeres. Formally, however, enzyme activation could occur at any time during the development of the immortal phenotype. An imbalance between telomerase activity and incomplete replication could also favour telomere elongation. Indeed, the TRFs of the 293(CSH) subline are twice as long as those of the parental line; long TRFs are also present in one of three sublines of HeLa cells (deLange *et al.*, 1990) and in a subset of colon mucosal tumours (Hastie *et al.*, 1990).

### Significance and implications

Our *in vitro* model of cellular transformation can be summarized as follows. Growth control of normal somatic cells is overridden by oncogene expression and the replicative limit of these cells is extended. In the absence of telomerase, telomeres shorten until cells become nonviable (crisis), presumably due to chromosome instability. If an additional mutation activates telomerase, an immortal clone may survive the crisis period with stabilized telomeres.

This model may have direct relevance to tumourigenesis *in vivo*. For example, the finite lifespan of partially transformed (pre-immortal) cells which lack telomerase might explain the frequent regression of tumours after limited growth *in vivo*. In bypassing the checkpoint representing normal replicative senescence, transformation may confer

an additional 20−40 population doublings during which an additional ≈2 kbp of telomeric DNA is lost. Since 20−40 doublings ($10^6-10^{12}$ cells in a clonal population) potentially represents a wide range of tumour sizes, it is possible that many benign tumours may lack telomerase and naturally regress when telomeres become critically shortened. We predict that more aggressive, perhaps metastatic tumours would contain immortal cells which express telomerase. To test this hypothesis, we are currently attempting to detect telomerase in a variety of tumour tissues and to correlate activity with proliferative potential. Anti-telomerase drugs or mechanisms to repress telomerase expression could be effective agents against tumours which depend upon the enzyme for maintenance of telomeres and continued cell growth.

# Materials and methods

## Cells and transfection

Human embryonic kidney (HEK) cells obtained by trypsinization of fetal organs were seeded at high density, harvested when confluent, frozen and stored in liquid nitrogen. Upon thawing, cells were seeded at approximately half confluence and were grown to confluence in α-MEM with 10% fetal calf serum. This stage of growth was arbitrarily denoted 0 MPD. For transfections, cells were seeded at a density of $2.5 \times 10^5$ cells/100 mm plate and grown for 24 h. Transfection with pSV3neo [encoding the SV40 early region and the bacterial neomycin gene (Southern and Berg, 1982)] was performed by the calcium phosphate technique (Graham and van der Eb, 1973). The cells were incubated for 8 h in the presence of the DNA−calcium phosphate precipitate, refed with fresh medium and 48 h after transfection were reseeded at low density in G418-containing medium. Eleven independent colonies (HA1-IM and HA2-, HA3-, HA4- and HA5-EL: Stewart and Bacchetti, 1991; HA6-, HA7-, HA8-, HA9-, HA10- and HA11-EL: LeFeuvre,C.E. and Bacchetti,S., unpublished) surviving G418 selection were isolated and expanded. All of the cells had altered morphology and expressed SV40 tumour antigens. Cells were subcultured when confluent and reseeded at a split ratio of 1:4 or 1:8 in α-MEM with 10% fetal calf serum. Thus each successive passage represents two or three MPD. In all populations cell viability remained high up to the onset of crisis, as indicated by their replating efficiency and stable growth rate. Five of the eleven transformed populations generated from kidney cells from two different donors were chosen for in depth analysis. Aliquots of the 293 cell population frozen prior but close to crisis and of the immortal (post-crisis) 293 cell line (Graham *et al.*, 1977) were obtained from F.L.Graham (McMaster University). These cells were grown in monolayers in MEM-F11 plus 10% newborn calf serum. The 293 cell line adapted for growth in suspension, 293(CSH), was obtained from B.Stillman (Cold Spring Harbor Laboratory). These cells and the 293-N3S subline derived from passage in mice (Graham, 1987) were grown in spinner flasks in Joklik's modified MEM with 10% horse serum. All three lines were used for DNA analysis and assayed for telomerase activity.

## DNA extraction

Cells were lysed and proteins were digested in 10 mM Tris−HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K at 48°C overnight. Following two extractions with phenol and one with chloroform, DNA was precipitated with ethanol and dissolved in 10 mM Tris−HCl (pH 8.0) and 1 mM EDTA (TE).

## Determination of TRF length and amount of telomeric DNA

Genomic DNA was digested with *Hin*fI and *Rsa*I, extracted and precipitated as above and redissolved in TE. The DNA concentration was measured by fluorometry (Morgan *et al.*, 1979). DNA samples (1 μg each) were loaded onto a 0.5% agarose gel and electrophoresed for 13 h at 90 V. The gel was dried at 60°C for 30 min, denatured in 1.5 M NaCl and 0.5 M NaOH for 15 min, neutralized in 1.5 M NaCl, 0.5 M Tris−HCl (pH 8.0) for 10 min and hybridized to a 5′-[$^{32}$P](CCCTAA)$_3$ telomeric probe in 5× SSC (750 mM NaCl and 75 mM sodium citrate), 5× Denhardt's solution (Maniatis *et al.*, 1982) and 0.1 × P wash (0.5 mM pyrophosphate, 10 mM Na$_2$HPO$_4$) at 37°C for 12 h. Following three high stringency washes in 0.1× SSC at 20−22°C (7 min each), the gel was autoradiographed on pre-flashed (OD = 0.15) Kodak XAR-5 X-ray films for 24−48 h with enhancing

screens. Each lane was scanned with a densitometer and the data were used to determine the amount of telomeric DNA and the mean TRF length as previously described (Harley *et al.*, 1990).

## Cytogenetic analysis

Cells were seeded at a 1:8 split ratio onto plates containing coverslips and grown to half confluence. Following addition of colcemid (0.1 μg/ml) for 5 h to arrest chromosomes at metaphase, the cells were incubated in hypotonic KCl (0.075 M) at 37°C and fixed in cold methanol:acetic acid (3:1 v/v). Chromosome were stained with 5% Giemsa and metaphases were scored regardless of ploidy, unless chromosome overlaps precluded analysis. Aberrations were identified according to Buckton and Evans (1973).

## Preparations of S-100 cell extracts

Approximately $6 \times 10^8$ cells were used for each extracts. Cells growing in suspension were collected by centrifugation for 10 min at 1800 r.p.m. (500 *g*) at 4°C with no brake in a Beckman JA-10 fixed angle rotor. Cells growing in monolayer were harvested by scraping with a rubber policeman and centrifuged as above. The pellets were rinsed twice in cold PBS (2.7 mM KCl, 1.5 mM KH$_2$PO$_4$, 137 mM NaCl and 8 mM Na$_2$HPO$_4$) followed by centrifugation for 3 min at 2000 r.p.m. (570 *g*) at 4°C in a swing-out rotor. The final pellet was rinsed in cold 2.3× Hypo buffer (1 × Hypo buffer: 10 mM HEPES pH 8.0, 3 mM KCl, 1 mM MgCl$_2$, 1 mM DTT, 0.1 mM PMSF, 10 U/ml of RNasin, 1 μM leupeptin and 10 μM pepstatin A; in some experiments the latter two reagents were omitted), centrifuged for 5 min and resuspended in 0.75 vol of 2.3× Hypo buffer. After incubation on ice for 10 min the sample was transferred to an ice cold 7 ml Dounce homogenizer and homogenized on ice using a B pestle (25−55 μm clearance). After a further 30 min on ice the sample was centrifuged for 10 min at 10 000 r.p.m. (16 000 *g*) at 4°C in a Beckman J3−13.1 swing-out rotor. One-fiftieth volume of 5 M NaCl was added and the sample was centrifuged for 1 h at 38 000 r.p.m. (100 000 *g*) at 4°C in a Beckman Ti50 rotor. Glycerol was added to a final concentration of 20% and the extract aliquoted and stored at −70°C. Protein concentration in a typical extract was ≈4 mg/ml.

## Telomerase and DNA polymerase assays

Telomerase activity was assayed by a modification of the method of Morin (1989). Aliquots (20 μl) of S-100 cell extract were diluted to a final volume of 40 μl containing 2 mM dATP, 2 mM dTTP, 1 mM MgCl$_2$, 1 μM (TTAGGG)$_3$ primer, 3.13 μM (50 μCi) [α-$^{32}$P]dGTP (400 Ci/mmol), 1 mM spermidine, 5 mM β-mercaptoethanol, 50 mM potassium acetate and 50 mM Tris−acetate (pH 8.5). In some experiments reaction volumes were doubled. The reactions were incubated for 60 min at 30°C and stopped by addition of 50 μl of 20 mM EDTA and 10 mM Tris−HCl (pH 7.5) containing 0.1 mg/ml RNase A, followed by incubation for 15 min at 37°C. To eliminate proteins, 50 μl of 0.3 mg/ml proteinase K in 10 mM Tris−HCl (pH 7.5), 0.5% SDS was added for 10 min at 37°C. Following extraction with phenol and addition of 40 μl of 2.5 M ammonium acetate and 4 μg of carrier tRNA, the DNA was precipitated with 500 μl of ethanol at −20°C. DNA pellets were resuspended in 3 μl of formamide loading dye, boiled for 1 min, chilled on ice and loaded onto an 8% polyacrylamide−7 M urea sequencing gel and run at 1500 V for 2.5 h using 0.6× TBE buffer. Dried gels were exposed to Kodak XAR-5 pre-flashed film at −70°C with enhancing screen. Typical autoradiograph exposures were between 2 and 7 days. DNA polymerase was assayed according to Bauer *et al.* (1988) in 25 μl reactions containing 12.5 μl of S-100 extract and 20 mM Tris−HCl pH 7.8, 8 mM MgCl$_2$, 0.5 mg/ml BSA, 5 mM DTT, 2 mM spermidine, 80 μM dGTP, TTP and dCTP, 20 μM dATP, 4% glycerol, 0.5 μCi [α-$^{32}$P]dATP (3000 Ci/mmole) and 4 μg of high molecular weight gapped calf thymus DNA (Spanos *et al.*, 1981). Following incubation at 37°C for 30 min and addition of 10 μl of 0.5 M EDTA, samples were spotted in duplicate on DE81 paper. Percentage incorporation was calculated from the ratio of radioactive counts on paper washed extensively in 0.5 M Na$_2$HPO$_4$, water and ethanol to input counts on unwashed paper.

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