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Stabilization of Short Telomeres and Telomerase Activity Accompany Immortalization of Epstein-Barr Virus-Transformed Human B Lymphocytes

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We have measured telomere length and telomerase activity throughout the life span of clones of human B lymphocytes transformed by Epstein-Barr virus. Shortening of telomeres occurred at similar rates in all populations and persisted until chromosomes had little telomeric DNA remaining. At this stage, some of the clones entered a proliferative crisis and died. Only clones in which telomeres were stabilized, apparently by activation of telomerase, continued to proliferate indefinitely, i.e., became immortal. Since loss of telomeres impairs chromosome function, and may thus affect cell survival, we propose that telomerase activity is required for immortality. We have now detected this enzyme in a variety of immortal human cells transformed by different viruses, indicating that telomerase activation may be a common step in immortalization.

Transformation by DNA tumor viruses usually confers on cultured human cells an extended life span (reviewed in references 3, 30, 33, and 34). In the case of simian virus 40 (SV40), adenovirus, and human papillomavirus, most transformed populations eventually reach a proliferative crisis and perish (13, 14, 32), only rarely yielding immortal clones. The low efficiency of immortalization by these viruses (14, 20, 28, 32, 34) has suggested that viral proteins are insufficient for this process and that additional events, such as mutations of host genes, are required (29, 34, 35, 37). In contrast, Epstein-Barr virus (EBV) (23, 34) and polyomavirus (34, 35) reportedly immortalize human cells at high frequency with no detectable crisis, suggesting that these viruses may encode most, or all, of the functions required for immortalization (34).

Cell life span and telomere length appear to be related. Telomeres are the most distal structures of chromosomes, composed of highly repetitive DNA, $(TTAGGG)_n$ in humans (6, 11), and protein, which together maintain the stability of chromosome ends (reviewed in references 4, 5, and 44). In cells transformed by SV40 and adenovirus type 5 (9) or by human papillomavirus type 16 (21a, 32), telomeres shorten until crisis, at which point little or no telomeric DNA remains. Concomitantly, dicentric chromosomes increase in frequency, compatible with loss of functional telomeres and fusion of chromosomal ends (9). After crisis, telomere shortening is arrested, apparently by activation of telomerase (9), the enzyme which elongates telomeres de novo (15, 16, 26, 43), and frequency of dicentric chromosomes stabilizes (9). On the basis of these observations, we proposed that virus transformation overcomes the checkpoint regulating the life span of normal cells (senescence) but does not prevent loss of telomeric DNA. Telomeres eventually reach a critically short length, leading to chromosome rearrangements and finally to cell death. Only by stabilizing telomere length by addition of telomeric repeats through activation of telomerase can cells continue to proliferate (9, 18).

Given the ability of EBV and polyomavirus to immortalize cells at high efficiency and apparently in the absence of proliferative crisis, we speculated that activation of telomerase in cells transformed by these viruses may occur early, circumventing the possible consequences of telomere loss. To investigate this hypothesis, we measured telomere length, chromosome aberrations, and telomerase activity throughout the lifespan of clonal populations of EBV-transformed human B lymphocytes. As with other viruses (9, 32), we find, however, that reactivation of telomerase is a late event in the generation of immortal cells, and that the presence of critically short telomeres correlates with proliferative crisis of nonimmortal cells. Shortening of telomeres during growth of EBV-transformed lymphocytes has recently been reported by others (17).

Growth properties of EBV-transformed clones. Human B lymphocytes from two different donors were infected with EBV (strain B95-8), and independent clonal populations were isolated and subcloned (38). The latter populations were provided to us at early passage by B. Sugden (McArdle Laboratories, University of Wisconsin) and were grown in RPMI with 10% fetal calf serum and antibiotics and, for ≈5 population doublings (pd), were maintained on human fibroblast feeder layers (38). Two of the clones, B2 and B5, ceased proliferating at pd 45 and 95, respectively (Table 1), at what appeared to mimic the proliferative crisis of adenovirus type 5- and SV40-transformed cells (13, 14). This event was reproducible, as shown by culturing of B2 a second time from frozen early-passage cells. Loss of cell viability was likely not due to activation of the lytic cycle since EBV-transformed populations release very little virus and maintain a constant copy number of viral genomes throughout their life span (36, 39). Our data suggest that proliferative crisis does occur in EBV-transformed cells and is detectable when clonal populations are analyzed. This crisis is likely masked in nonclonal populations because of the high rate of immortalization by EBV (23) (Table 1).

Clones B3 and B4 reproducibly yielded immortal populations (Table 1). The doubling time of these cultures did not

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TABLE 1. Properties of EBV-transformed B lymphocytes

Clone	Lifespan (pd)[a]	ΔTRF (bp/pd)	TRF length at crisis (kbp)	Telomere length at crisis (kbp)[b]	Dicentrics[c]			Telomerase activity[d]	
					Precrisis	Crisis	Postcrisis	Precrisis	Postcrisis
B2	Mortal, crisis at pd 45	−120	4.5	ND[e]	−	ND	ND	−	ND
B3	Immortal, crisis at pd 60–100[f]	−100	3.5	1.5	−	++	−	−	+
B4	Immortal, crisis at pd 70–100	−90	3.5	2.0	−	+	−	−	+
B5	Mortal, crisis at pd 95	−80	5.0	ND	−	++	ND	−	ND
Mean (± SD)		−100 ± 20	4 ± 1	1.8 ± 0.1					

[a] Life span is recorded as pd after isolation of subclones. Crisis was defined as the time when cell death exceeded cell proliferation (B2 and B5) or when cell viability decreased, as determined by trypan blue exclusion (B4). Immortal clones were cultured for more than 150 pd.

[b] Amount of telomeric $(TTAGGG)_n$ DNA at crisis was calculated, as previously described (9, 22), by comparing the rate of loss of hybridization signal (Fig. 1C) with the rate of decrease in TRF length (Fig. 1B), and by assuming that decrease in both parameters is due exclusively to loss of $(TTAGGG)_n$ repeats.

[c] A minimum of 50 metaphases was examined at different time points before, during, and after crisis. The average number of dicentrics per 50 metaphases in each period was as follows: 0 to 1 (−), 2 to 4 (+), and more than 4 (++).

[d] Extracts were considered positive (+) if the 6-nucleotide pattern was detected in a 4-day exposure and negative (−) if no pattern was detected in a 10-day exposure.

[e] ND, not determined.

[f] Cell viability was not measured for clone B3, and crisis was defined as the period in which TRFs were critically short and the frequency of dicentrics dramatically increased.

vary appreciably with age; however, in the case of B4 cells, for which viability was measured, there was a detectable increase in the number of nonviable cells (5 to 12%) between pd 70 and 100 (Table 1). Whether this represents crisis is unclear. Even in SV40 and adenovirus transformants, the severity of crisis can vary, and immortalization with little or no overt crisis has occasionally been detected (7, 9, 24, 31).

Telomerase activity is detected in immortal populations concomitantly with stabilization of telomeres. To measure telomere length as a function of cell age, genomic DNA was isolated at regular intervals throughout the life span of each of the clones. The DNA was digested with restriction enzymes *Hin*fI and *Rsa*I which liberate terminal restriction fragments (TRFs), composed of telomeric TTAGGG repeats and of subtelomeric DNA containing short stretches of TTAGGG DNA, degenerate TTAGGG, and unique repeats (1, 12). Digested DNA was resolved in agarose gels and hybridized with the telomeric probe $^{32}P(CCCTAA)_3$. Gels were stringently washed and exposed to PhosphorImager screens (Molecular Dynamics) to visualize the TRFs, as described elsewhere (2, 9). In all populations, TRFs had heterogeneous distributions (Fig. 1A) since their length varies between different chromosomes and even the same chromosomes in different cells (9, 12, 22). Distinct DNA fragments of 2 kbp and smaller (Fig. 1A) were present in all clones. These species were resistant to exonuclease *Bal* 31 (not shown) and thus likely correspond to nontelomeric interstitial TTAGGG repeats (2, 9, 40, 44). In addition, DNA from B3 and B4 cells contained a unique fragment of 4 kbp which disappeared beyond pd 44 to 47 (Fig. 1A). This suggests that cells containing this polymorphic sequence were lost from the populations. Since B3 and B4 were derived from the same donor and cloned twice, this polymorphism may have been present in the parental cells. Its loss is consistent with the occurrence of genetic heterogeneity in transformed populations, as indicated by the existence of polyploid cells (see below).

As shown in Fig. 1A for B3, both TRF length and intensity of telomeric signal decreased with cell age, indicating an actual loss of TTAGGG repeats (9, 19). Similar results were obtained for all the clones and are plotted as TRF length and telomeric signal versus pd in Fig. 1B and C. Specifically, the rate of decrease in TRF length was, on average, 100 bp/pd (Table 1), similar to previous reports (2, 9, 40). In the terminal passages of B2 and B5 (pd 45 and 95, respectively), TRF length was ≈4.7 kbp, on average (Table 1; Fig. 1B). On the basis of the data for B3 and B4 (Table 1), a TRF length of 4.7 kbp corresponds to a calculated average length of <2 kbp of residual true telomeric DNA (TTAGGG). TRF length is quite heterogeneous, and thus, it is likely that some chromosomes in these populations actually lack telomeric DNA (9). Indeed, since the residual hybridization signal may be due to subtelomeric TTAG GG repeats, many chromosome ends may be composed of nontelomeric DNA. The actual sequence at the termini of chromosomes appears to be critical since single-nucleotide permutations of the telomeric repeat cause cell senescence and death in *Tetrahymena thermophila* (43). This suggests that shortening of telomeres may not be detrimental per se until a chromosome lacks TTAGGG repeats at its end, in agreement with the observation that large terminal deletions are tolerated only if broken ends acquire telomeric DNA (5, 21, 42–44). In clones B3 and B4, TRFs reached their shortest length of 3.5 kbp by pd 100 and thereafter were stable. It is intriguing that the limited decrease in cell viability occurred in B4 cells between pd 70 and 100. It is also interesting to note that the difference in initial telomere length in B5 compared with B2 (≈13 versus ≈10 kbp [Fig. 1B]) may account for the longer replicative capacity (45 pd longer) of this clone, as suggested for cultured human fibroblasts (2).

To assay telomerase activity, extracts were prepared by collecting 10^7 to 10^8 cells in mid-log phase from each of the clones before and, if applicable, after crisis. S100 extracts were prepared either as previously described (9, 10) or by first washing 10^7 cells in 1 ml of hypotonic buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol), before permeabilization in 200 μl of lysis buffer (10 mM Tris-HCl [pH 8], 1 mM $MgCl_2$, 10% glycerol, 5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.4% Nonidet P-40) as described by Wang et al. (41). Telomerase was assayed by incubating at 30°C for 1 to 2 h 20 μl of extract with buffer (10, 11) or 10 μl of extract with an equal volume of 2× buffer {100 mM Tris-HCl [pH 8], 6 mM $MgCl_2$, 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], 2 mM spermidine, 0.2 mM spermine, 2 mM β-mercaptoethanol, 2 μM $(TTAGGG)_3$, 5 mM dATP, 5 mM TTP, and 1.25 μM [α-^{32}P]dGTP [10 mCi/ml] [41]}. Reactions were terminated by digestion with proteinase K, followed by phenol and chloroform extractions and removal of unincorporated nucleotide by Sephadex GS-50 spin columns (9, 10). DNA was precipitated, washed with ethanol, resuspended in loading buffer, and resolved on sequencing gels which were then exposed to PhosphorImager screens (9, 10). Since the enzyme contains an RNA template (16, 26, 43), RNase was added to duplicate reaction mixtures as a control prior to the addition of [α-^{32}P]dGTP. This treatment abolishes the 6-nucleotide ladder

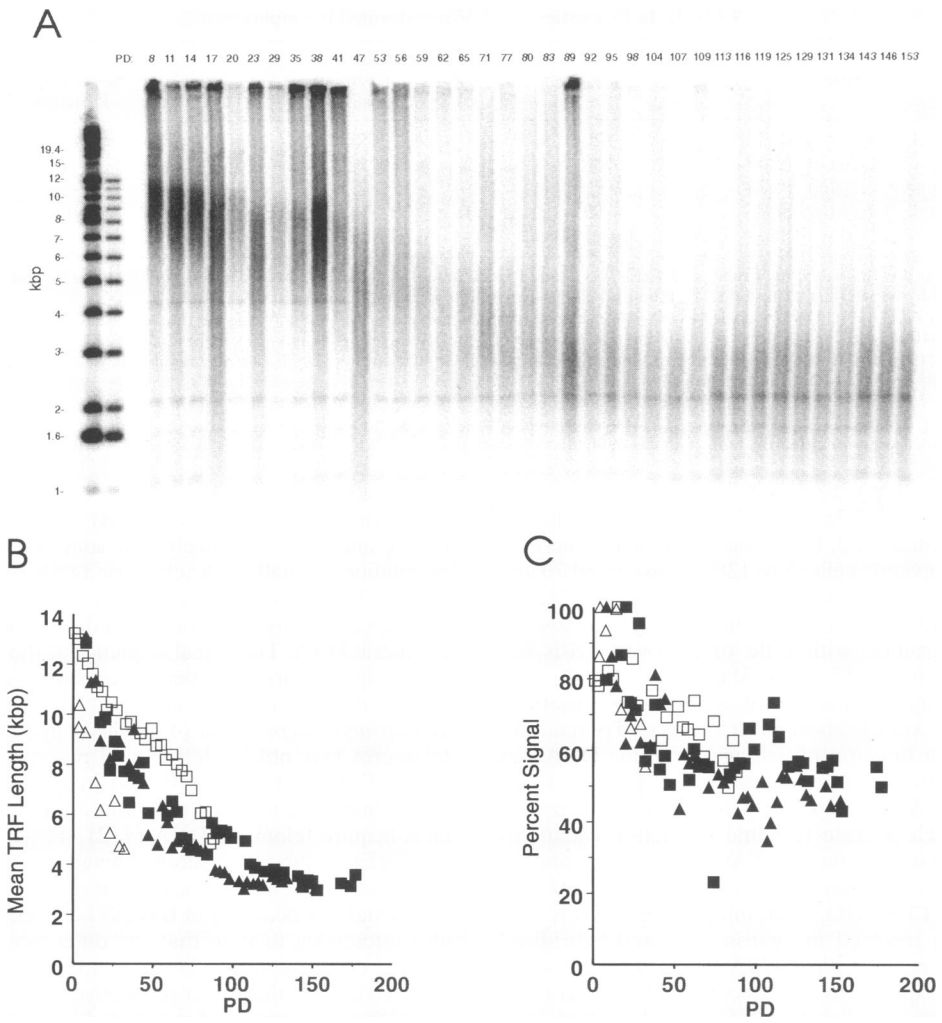

FIG. 1. TRF length and amount of telomeric DNA (intensity of hybridization signal) in clonal populations of EBV-transformed B lymphocytes. (A) Southern hybridization of the telomere probe $(CCCTAA)_3$ to *Hin*fI- and *Rsa*I-digested DNA from clone B3 at the indicated pd. (B and C) The TRF distribution was quantified from 21 to 2 kbp (2, 9) for a minimum of three separate experiments for the mortal clones B2 (△) and B5 (□) and the immortal clones B3 (▲) and B4 (■). The values were used to determine mean TRF length (B) and signal intensity, expressed as percentage of the maximum signal (C).

characteristic of the pausing of telomerase during elongation of a primer (9, 16, 26, 43).

An RNase-sensitive 6-nucleotide ladder comigrating with an identical pattern from telomerase-positive 293 cell extracts (9) was detected in extracts from the immortal populations derived from clones B3 and B4 (Fig. 2; Table 1). No activity was detected, even in longer exposures, in either of these clones or in the mortal clones B2 and B5 before crisis (Fig. 2; Table 1) and in peripheral blood leukocytes from normal donors (10). All extracts were prepared from the same number of cells and had similar protein concentrations and DNA polymerase activity (not shown). Lack of detectable telomerase activity in precrisis and control cells was confirmed by using both methods for preparation and testing of extracts (9, 10, 41). We occasionally detected an RNase-sensitive 5-nucleotide ladder (not shown) or an RNase-insensitive single-nucleotide ladder (Fig. 2) but found that these patterns were not template-dependent when permutated oligonucleotides were used, indicating that they were not due to telomerase (8, 26, 27). Thus, as in the case of cells transformed by other viruses (9), telomerase activation or up-regulation is not an early event in EBV transformants but occurs when telomere length is stabilized near crisis. The existence of stable but short telomeres in immortal populations, moreover, argues against selection of preexisting telomerase-positive cells, since the presence of these cells at early passages should yield immortal clones with long stable telomeres.

Cytogenetic properties of transformed B lymphocytes. SV40-transformed cells exhibit a dramatic increase in dicentric chromosomes at crisis (9, 25, 30, 37), possibly because of the loss of functional telomeres (9, 18). To determine whether dicentrics also form at crisis in EBV-transformed cells, metaphase spreads were prepared at regular intervals throughout the life spans of the populations and scored for chromosomal aberrations and ploidy (37). All populations contained a fraction of polyploid cells, except for B3 which was almost exclusively polyploid. The incidence of aberrations was very low throughout the life span of all clones (<2/50 metaphases, on average, excluding dicentrics) compared with SV40-transformed cells (9, 25, 30, 37), although in some cases aberrations

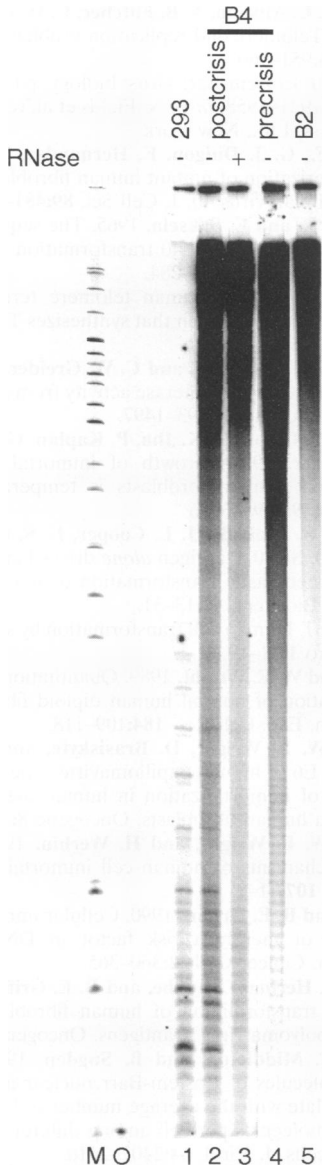

FIG. 2. Telomerase activity in EBV-transformed B lymphocytes. S100 extracts from the telomerase-positive cell line 293 (lane 1), the immortal clone B4 after and before crisis (lanes 2 to 4), and the mortal clone B2 before crisis (lane 5) were incubated with buffer, TTP, dATP, the telomeric oligonucleotide $(TTAGGG)_3$, and [α-^{32}P]dGTP. As a control, RNase was added to the B4 postcrisis extract prior to addition of [α-^{32}P]dGTP (lane 3). Reaction products were resolved on a sequencing gel and detected after exposure to a PhosphorImager screen (Molecular Dynamics) for 6 days (9, 39). M, molecular weight marker; O, $^{32}P(TTAGGG)_3$ oligonucleotide.

increased near crisis. Few or no dicentrics were detected in transformed populations at early times when telomeres were long (Fig. 3; Table 1). Interestingly, however, when telomeres became critically short, the frequency of dicentrics increased dramatically, at least in B5 cells at terminal passage and in B3 cells between pd 40 and 80 (Fig. 3; Table 1). Thus, as in the case of SV40 transformants (9), dicentric formation may correlate with critically short telomeres, reduced cell viability, and absence of telomerase activity. These observations agree with data from a variety of systems supporting the notion that

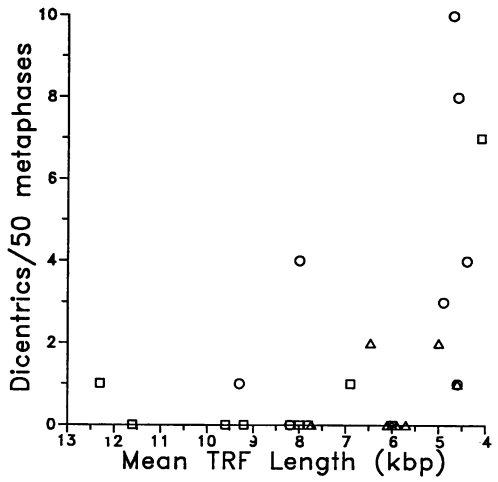

FIG. 3. Dicentrics per 50 metaphases versus mean TRF length in EBV-transformed B lymphocytes: B5 (□), B4 (△), and B3 (○). The number of dicentrics detected at crisis, when TRFs are 4 to 5 kbp in length, was found to be significantly different from the values corresponding to TRFs 1 kbp longer (5 to 6 kbp; $P = 0.004$) or to all larger TRFs (up to 13 kbp; $P < 0.0001$).

lack of telomeric DNA leads to highly recombinogenic chromosomes (4, 5, 9, 21, 44). Since EBV transformants contained few aberrations which could serve as precursors for dicentrics (e.g., breaks), it is tempting to speculate that the latter structures may result exclusively from fusion of chromosomes lacking telomeres. After crisis, only one dicentric was detected in B3, and none were detected in B4 cells, out of 150 and 300 metaphases, respectively. Telomerase presumably balances telomere shortening by addition of TTAGGG repeats to chromosome ends, thus potentially restoring telomere function (chromosome stability). Lack of dicentrics in immortal populations may be a consequence of this process.

Activation of telomerase may be a common step in the acquisition of immortality. Clonal populations of EBV-transformed B lymphocytes undergo a proliferative crisis, indicating that the virus does not encode all the necessary functions required for immortalization. Presumably, the remaining events involve mutations of host genes. It is clear that the high rate of immortalization by EBV (23) relative to SV40 and adenovirus is not due to early activation of telomerase, since neither stable telomeres nor telomerase activity were detected until after pd 80. Perhaps EBV can directly induce more of the events required for immortalization than other viruses, or immortalization efficiency is dependent upon the cell type. As in the case of SV40- and adenovirus type 5-transformed cells, in EBV transformants telomere shortening, and potentially crisis, are overcome concomitantly with detection of telomerase activity, suggesting that this enzyme may be universally required for immortality. We have recently detected telomerase in late-stage ovarian carcinomas (10). Our present and previous (9) results render virus-transformed cells a suitable system for the study of the enzyme in tumor development and the testing of telomerase-inhibitory drugs.

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