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Functional reintroduction of human telomeres into mammalian cells

(electroporation/chromosome breakage/*in situ* hybridization/histidinol selection)

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ABSTRACT **Telomeric sequences of eukaryotes consist of short tandem repeats organized in arrays of variable length in which the guanine-rich strand runs 5′ → 3′ toward the chromosomal end. The terminal repeats in yeast are the only elements necessary for telomere function in this organism. To test whether mammalian terminal repeats can function after reintroduction into a mammalian cell, a repeat-containing terminal fragment from a human chromosome was electroporated into a hamster–human hybrid cell line. In 6 of 27 independent transformants analyzed, the introduced sequences were found at the ends of chromosomes, based on all available criteria. Terminal restriction-fragment heterogeneity and the survival of these chromosomes demonstrate that these telomeres are functional. Cytogenetic evidence from one of these cell lines suggests that chromosome breakage with healing at the integration site is the mechanism responsible for the terminal location.**

In eukaryotes the functional elements known to be essential for the stable maintenance and transmission of chromosomes are origins of replication, centromeres, and telomeres. In yeast these three elements have been cloned and characterized (1–3), allowing minichromosomes to be constructed. The manipulation of artificial chromosomes has made it possible to explore the effects of the position and spacing of the various functional components (4) and to study the proteins that interact with them (5, 6). The development of yeast artificial chromosome cloning systems for large DNA fragments has been critically dependent on an understanding of these elements. As a mapping tool, homologous recombination has been used to target telomeric sequences to particular loci (7). Resolution and healing at these telomeres leads to chromosome breakage, which can be detected by pulsed-field gel electrophoresis (8).

In mammals the only one of these functional elements available is the telomere. The terminal repeats that characterize all known telomeres have a strand bias in G+C composition—the guanine-rich strand being orientated 5′ → 3′ toward the end of the chromosome (9). In mammals and other vertebrates this repeat sequence is TTAGGG (10, 11), and the ends of the chromosomes consist of arrays of this sequence, which vary in length from cell to cell (12, 13). Recently, these terminal fragments of the human genome have been selectively cloned (14–18) and found to support telomere function in yeast. Because these cloned human telomeres can function in yeast, it seemed probable that they would also have functional potential when reintroduced into mammalian cells.

To test this hypothesis a repeat-containing terminal fragment from a human chromosome was electroporated into a hamster–human hybrid cell line. The human telomeric DNA used in this study had been isolated previously by complementation of a deficient yeast artificial chromosome (16). The length of the tandem array of TTAGGG repeats in this clone is ≈500 base pairs (bp), considerably less than in the human genome, suggesting that the TTAGGG tract had been abbreviated during the cloning procedure (19). Telomere-containing vectors have been constructed based on the plasmid pSV2his (20), which confers histidinol resistance on mammalian cells. In addition to a human telomeric fragment, the vectors contain sequences from the human pseudoautosomal locus *MIC2* (21). These sequences provide a "tag," allowing for the subsequent cytogenetic localization of the integrated construct by *in situ* hybridization.

We have analyzed 27 histidinol-resistant transformants produced by random integration of the linearized construct after electroporation of a somatic cell hybrid, clone 2D, which contains a single human X chromosome. Six of these transformants carry the introduced sequence at the telomere of a chromosome, as determined by *in situ* hybridization and nuclease BAL-31 digestion. In three of these transformants this location was further confirmed by restriction site mapping.

MATERIALS AND METHODS

Recombinant DNA Constructs. Cloning manipulations were done by standard methods (22). A 7.5-kilobase (kb) *Eco*RI intronic fragment of the human *MIC2* locus was cloned into a unique *Eco*RI site in pSV2his (20). The yeast artificial chromosome-cloned human telomeric DNA had previously been cloned into Bluescript (pHutel-2-end) (19). The telomeric sequence starts with 12 bp of yeast terminal repeat, followed by ≈500 bp of the human simple telomeric-repeat sequence TTAGGG and related variants, such as TTGGGG. Adjacent to this abbreviated TTAGGG array is a region of 85% G+C content composed of 27 repeats of a 28-bp sequence. This region contains restriction sites for several rare cutter enzymes and is similar in structure to many minisatellite arrays. The remaining subtelomeric sequences in this clone display considerable internal repetition, including a region of 54-bp repeats. The 2-kb *Eco*RI–*Hin*dIII fragment of pHutel-2-end was blunt-ended by a Klenow fill-in reaction and ligated to the blunt-ended *Acc* I site of the construct. This construct (pHTM) can be linearized by *Nde* I, which cuts within the pBR322 sequences ≈50 bp away from the remains of the pHutel-2-end *Eco*RI site. The terminal repeats are orientated such that the guanidine-rich strand runs 5′ → 3′ away from the selectable marker *hisD*. pHTtkM3 differs from pHTM only in that it contains additional Bluescript polylinker sequences flanking the telomeric DNA plus a herpes simplex virus thymidine kinase gene and enhancer sequences cloned into the pBR322 *Nde* I site. pHTtkM3 can be linearized by *Xba* I (Fig. 1*a*).

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Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

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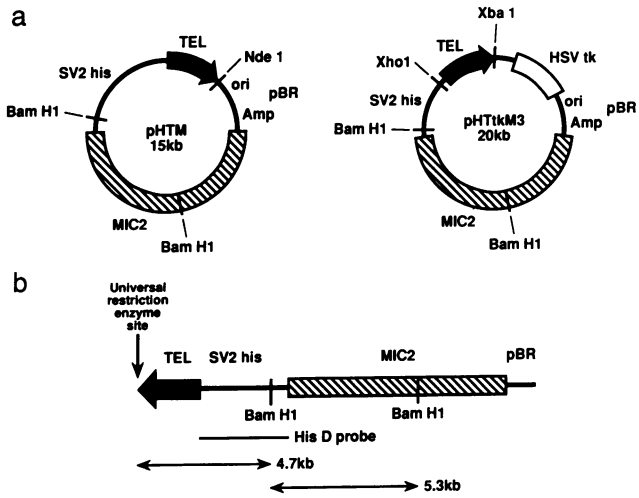

FIG. 1. (*a*) Constructs used for the reintroduction of human telomeric sequences (TEL) into mammalian cells: pHTM (linearized by digestion with *Nde* I) and pHTtkM3 (linearized by *Xba* I). (*b*) Simplified restriction map of the linearized constructs, showing the *Bam*HI sites and *hisD* probe used in the molecular analysis. ori, Origin of replication; Amp, ampicillin-resistance gene; SV2his, the bacterial *his* D gene under control of simian virus 40 early promoter.

Cell Culture and Electroporation. The somatic cell hybrid clone 2D (23) contains, as its only human contribution, a single intact X chromosome. The rodent parent, Wg3H, is a hypoxanthine phosphoribosyltransferase-deficient ($HPRT^-$) Chinese hamster cell line. The cells are routinely grown in Dulbecco's modified Eagle's medium/10% fetal calf serum/penicillin and streptomycin/100 μM hypoxanthine/1.6 μM thymidine/10 μM methotrexate (HAT). Cells were electroporated by using a Bio-Rad Gene Pulser apparatus. Actively growing cells were harvested by trypsinization, washed, and resuspended in phosphate-buffered saline (without Ca^{2+} or Mg^{2+}). Cells (1×10^6) were electroporated in a volume of 800 μl with 10 μg of linearized vector at 25 μF and 1.2 kV. Cell viability under these conditions is 40–60%. Cells were seeded into 75-cm^2 tissue-culture flasks (1×10^6 per 75 cm^2). Histidinol selection was applied after 24 hr. Histidinol-resistant (his^r) colonies were selected for 7–10 days in HAT medium/5 mM histidinol (Sigma). There were ≈500 his^r colonies per 75 cm^2 flask. The transformation frequency is therefore 1 in 10^3 of cells surviving electroporation. Individual colonies were picked for further analysis.

DNA Preparation, Digestion, and Analysis. DNA from cell lines and plasmids was prepared by standard methods (22). Southern transfer from agarose gels was done as described (19) onto nylon membranes (Hybond-N; Amersham). Nuclease BAL-31 (BRL) digestions were done in the manufacturer's recommended buffer at 30°C. Two-hundred micrograms of DNA was preincubated in 0.5 ml of BAL-31 buffer for 2 hr at 30°C. A sample of 0.1 ml was removed, and 8 units of BAL-31 was added. Further samples were removed and phenol-extracted at 5, 10, 20, and 40 min after enzyme addition. After phenol extraction and ethanol precipitation, samples were digested with *Bam*HI (Boehringer Mannheim) in the recommended buffer with 20 units of enzyme for 18 hr. DNA concentrations in the digests were measured by fluorimetry with Hoescht 33258, and 5-μg samples were loaded on a 0.8% agarose gel and electrophoresed for 600 V·hr in TAE (1× TAE = 0.04 M Tris acetate/1 mM EDTA) buffer before transfer to nylon membranes. Filters were hybridized in 7% $NaDodSO_4$/0.5 M $NaPO_4$ at 68°C for 18 hr. After being washed in 0.1× standard saline citrate (SSC) (1× SSC = 0.15 M sodium chloride/15 mM sodium citrate)/0.1% $NaDodSO_4$ at 68°C, filters were exposed to Kodak XAR-5 film for 17–72 hr.

***In Situ* Hybridization.** *In situ* hybridization to metaphase chromosomes from exponential cultures was as described (19, 24, 25). After the slides were counterstained with propidium iodide at 2 μg/ml, they were examined using a Bio-Rad laser-scanning-confocal microscope. The slides were restained with 4′,6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml) to give banded chromosomes, and photographs of each cell were karyotyped and compared with stored images to identify the chromosomes with telomeric or internal hybridization sites.

Recovery and Analysis of Sequences at Integration Sites. DNA from cell lines with telomeric sites of the pHTM construct was digested with *Eco*RI and ligated at a concentration of 5 μg/ml with T4 ligase (Boehringer Mannheim) in the manufacturer's recommended buffer. After recircularization the DNA was ethanol-precipitated and introduced by electroporation into *Escherichia coli*. Plasmid DNA recovered from ampicillin-resistant colonies was sequenced by using Sequenase (United States Biochemical) and a primer, CCTGATTCTGTGGATAACCG, derived from pBR322 sequence, starting at position 2454.

RESULTS

Molecular Analysis of Integrants. As an initial screen, DNA from 27 independent transformants was digested with *Bam*HI and analyzed by Southern blotting and probing with an *Acc* I–*Eco*RI fragment of pSV2his containing the *hisD* gene (Fig. 1*b*). Terminal restriction fragments from a functional telomere were expected to be heterogeneous as a result of the variation from cell to cell in the number of terminal repeats present. Of the 27 cell-line DNAs tested, 22 gave rise to two discrete fragments, 1 of which is common to all transformants and derived from the 5.3-kb *Bam*HI fragment shown in Fig. 1*b* (data not presented, and Fig. 2). The second fragment varied in size and represents the telomere-containing *Bam*HI fragment of the constructs. These lines were presumed to be nontelomeric integrants. The DNA from the remaining five cell lines showed a faint smear of hybridization in addition to the common band. This heterogeneous hybridization signal is characteristic of the terminal fragments derived from a functional telomere, and these cell lines were considered to be potential single-copy integrants in which the construct provides a functional telomere. These five cell-line DNAs and a number of DNAs from lines showing discrete bands were used for BAL-31 sensitivity assays, as described.

The enzyme BAL-31 degrades DNA exonucleolytically (26), and so a fragment of DNA present at a naturally occurring DNA end (a telomere) will be reduced in size before a more internal one. For an active telomere, the terminal fragment will be heterogeneous rather than discrete (13). Fig. 2 shows the results of BAL-31 digestion of DNA from three transformants. Two cell lines, HTtkM3 Cl70 and HTM18 Cl4, show heterogeneous smears of hybridization, which are reduced in size with increased periods of BAL-31 digestion. Before BAL-31 digestion the smear ranges in size from 5 to 7 kb. The corresponding fragment introduced is 4.7 kb (Fig. 1*b*), and the additional length of the heterogeneous smear represents terminal repeats added during telomere replication. The end product of this digestion appears as a band due to the presence of a G+C-rich region of human subterminal repeats that cause the enzyme to pause (19). One cell line, HTtkM3 Cl83, shows only discrete bands that are not reduced in size by BAL-31 digestion. All clones share the common fragment of 5.3 kb, which is not sensitive to BAL-31. This fragment would not be shortened by BAL-31 digestion until after the terminal fragment (this result is detectable in the 40-min time point of HTtkM3 Cl70). The presence of this fragment provides a control for the end specificity of BAL-31 digestion. All clones in which a smear of hybridization was detected in the primary screen exhibited the same

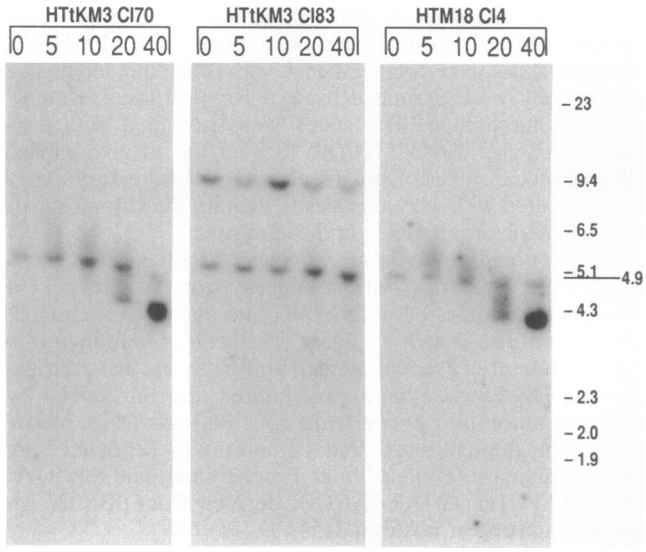

FIG. 2. BAL-31 digestions of two cell lines with functioning telomeric constructs (HTtkM3 Cl70 and HTM18 Cl4) and one interstitially located construct (HTtkM3 Cl83). High-molecular-weight DNA was digested with BAL-31 for the indicated times and digested with *Bam*HI after phenol extraction and ethanol precipitation. Hybridization is with the *hisD* probe. *Hin*dIII and *Eco*RI digests of lambda DNA are indicated as size markers (kb).

BAL-31 sensitivity of the smear. Conversely, no clones were found to have BAL-31-sensitive discrete bands. In three of the clones (HTM18 Cl4, HTM18 Cl8, and HTtkM3 Cl70), which showed a BAL-31-sensitive smear of hybridization, the terminal location of the integrated sequences was further confirmed by restriction mapping. The end of the telomere can, for mapping purposes, be regarded as a universal restriction site. Therefore, in any clone in which the inserted telomere is functional, a restriction analysis can be used to detect a series of heterogeneous DNA smears of increasing size (data not presented).

As a negative control, clone 2D cells were electroporated with pHTM vector from which all telomeric sequences had been deleted. The DNA from 26 histidinol-resistant colonies was digested with *Hin*dIII (which cuts within the vector MIC2 sequence) and subjected to Southern analysis with the *hisD* probe (data not shown). All 26 cell-line DNAs gave rise to discrete bands of hybridization. Therefore, in each of these cell lines the telomere-deleted construct has integrated interstitially and without providing telomere function.

Cytogenetic Analysis. To confirm the location of the exogenous DNA in these clones, 18 lines were analyzed by *in situ* hybridization. The results were scored blind, without knowledge of the results of molecular analysis. Representative spreads of two telomeric and two nontelomeric transformants and the parent hybrid line clone 2D are shown in Fig. 3. The probe used in these experiments was the larger *Xho* I–*Xba* I fragment of pHTtkM3 (Fig. 1*a*). This probe has homology to the *MIC2* locus, which is 2.5 megabase pairs from the pseudoautosomal telomere of the human X and Y chromosomes (21, 27, 28) and, therefore, provides an internal control for the *in situ* analysis. The human X chromosome of the cells is marked with an arrow in Fig. 3: the signal from the *MIC2* gene appears terminal in most cases. This result implies that a transgenome scored as subterminal would have to be at least 2.5 megabase pairs from the end of the chromosome. Five lines scored as terminal by molecular criteria were also scored as cytologically terminal. All but one of the remaining lines were nontelomeric by both criteria. This cell line, HTM10 Cl18, has cells with two different chromosomes carrying the transgenomes. One, which occurs in 25% of cells, is a submetacentric chromosome with the transgenome close to the centromere. The other (in 75% of cells) is an acrocentric chromosome with the transgenome located close to the centromere. DAPI banding of spreads after *in situ* hybridization is shown in Fig. 4 and demonstrates that the q arms of these chromosomes are identical. Although this cell line could be a mixture of two independent clones that have

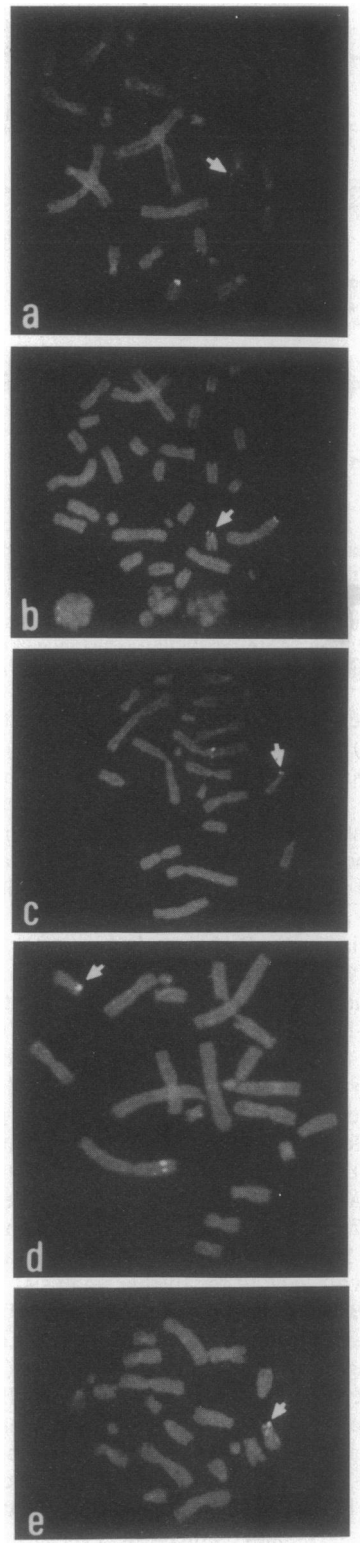

FIG. 3. Representative spreads of four cell lines and clone 2D after *in situ* hybridization. An arrow marks the human X chromosome. (*a* and *b*) Telomeric transformants. (*c* and *d*) Nontelomeric integrants. (*e*) Parent hybrid line, clone 2D.

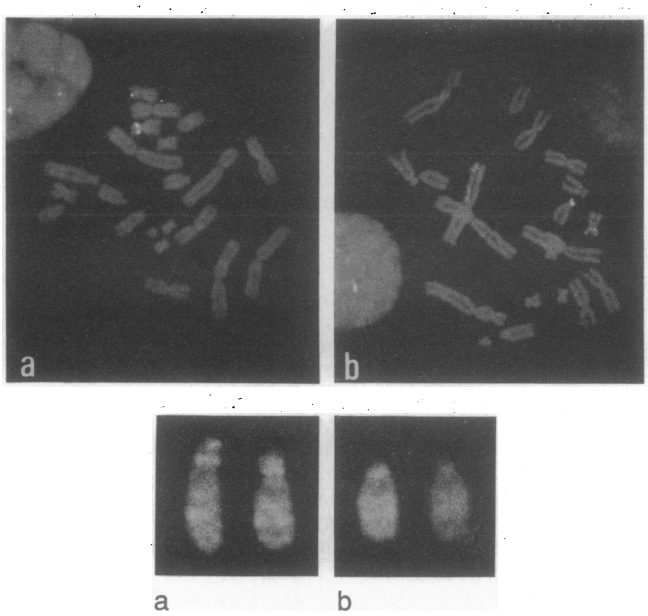

FIG. 4. *In situ* hybridization (*Upper*) and DAPI banding (*Lower*) of the two types of cells present in cell-line HTM10 Cl18. (*a*) A submetacentric chromosome with the transgenome close to the centromere. The hybridization signal is located between the two bright bands at the top of the DAPI-banded chromosome (representative of 25% of cells). (*b*) An acrocentric chromosome (in the remaining 75% of cells) with the transgenome located close to the centromere.

both suffered integrations in the same chromosome, a simpler explanation is that the clone with the centric fragment is derived from the clone with the subterminal integration. On Southern blot analysis, this cell line shows a single discrete fragment, as predicted for a single integration event; the expected smear of hybridization from those cells, which have a cytologically terminal fragment, is barely detectable.

Molecular Analysis of Chinese Hamster Sequences at the Integration Sites. To recover proximal Chinese hamster DNA sequences the structure of the pHTM construct was exploited. *Eco*RI digestion of cell-line DNAs containing this construct gives a fragment that contains the pBR322 amp^r gene and the origin of replication, thus allowing for plasmid rescue. Plasmids were recovered from three cell lines (HTM10 Cl37, HTM18 Cl4, and HTM18 Cl8), which have telomeric integration sites. The size of the plasmids corresponded to the size of the *Eco*RI restriction fragment from the corresponding cell line that was homologous to the amp^r-containing *Eco*RI–*Nde* I fragment of pBR322. The Chinese hamster DNA present in the clones in each case detected (on Southern blots) a new fragment only in the cell DNA from which it was derived and a fragment, or fragments, common to all cell lines. The fragments detected by the hamster-derived DNA from the three plasmids all differed in size (data not shown). To eliminate the possibility that the immediately flanking sequences contained terminal repeats, these plasmids were partially sequenced from a site in the pBR322 DNA close to the *Nde* I site at which the constructs were linearized. The sequences derived (GenBank numbers M60771, M60772, and M60773) showed no homology with each other or with other sequences in the data base and do not contain the terminal repeat sequence TTAGGG.

DISCUSSION

Telomeric DNA sequences appear highly conserved in structure throughout eukaryotes (29). These sequences occur as arrays of tandemly repeated sequences with a strand bias in G+C composition, and this structural conservation is reflected in a functional conservation. Telomeres from a number of species can function in the yeast *Saccharomyces cerevisiae* (29, 30). Human telomeres have been isolated by exploiting the ability of the yeast cell to use telomeric sequences derived from other organisms (14–17). In the present study a human telomere, originally cloned in yeast, has been reintroduced into mammalian cells by electroporation. Constructs containing a correctly orientated human telomeric fragment can form new telomeres at a high frequency. Of the 27 cell lines examined, 6 had a chromosome for which one telomere was provided by the construct we had introduced.

The construct transfected into these cells contains ≈500 bp of terminal-repeat sequence, which consists not only of TTAGGG repeats but also of related variants, such as TTGGGG. This short imperfect array is therefore sufficient for recognition by the cellular machinery responsible for telomere maintenance. The additional telomeres demonstrate terminal heterogeneity with a range of array lengths between 1 and 3 kb. The corresponding terminal array from the construct is 0.5 kb in length, and so this heterogeneity cannot be caused by shortening processes but must be a product of telomerase activity (31) or recombination (32). Several mechanisms could explain the generation of these new telomeres. Resolution of an integrated telomere is one possibility. Direct evidence for this mechanism comes from cytogenetic analysis of cell line HTM10 Cl18, in which a chromosome with an interstitial site is present in 25% of the cells, and an apparent centric product of breakage at this site occurs in the remaining 75% of cells. In yeast, head-to-head terminal-repeat arrays are processed at a rate of 1.1×10^{-2} per cell division to give new telomeres (33). However, electroporation of mammalian cells does not lead to the integration of multiple copies of the transfecting DNA at a high frequency (34). Telomere formation may be limited to those cells in which such an event has occurred. If a single copy of the construct is sufficient for generation of a functional telomere, those cell lines that have cytologically interstitial transgenomes may have lost the terminal repeats, so that the chromosomes are no longer subject to breakage. An alternative explanation is that the terminal repeats are retained, and the chromosomes break at a rate that varies from line to line. Internal arrays of $(TTAGGG)_n$ present in many mammalian genomes (35) are not subject to breakage at a detectable frequency, and the position of these arrays may be significant. Cell lines such as this may provide a model for fragile sites, which, it has been speculated, may be composed of simple repeated sequences like telomeres (36, 37).

Another mechanism is that the telomeric construct is targeted to existing telomeres. However the frequency of events that would have to be explained by homologous recombination (22%) is too high for this mechanism to be a convincing explanation (38). Moreover, a simple homologous recombination event between the chromosomal terminal repeat arrays and those present in the construct would have no overall effect and would not be detectable. However, such an event between the chromosomal TTAGGG repeats and either recircularized vector or multiple vector sequences aligned in a tandem array would effect insertion of the construct into an already functional telomere. The analysis of DNA immediately proximal to these new telomeres allows us to discriminate between breakage and homologous recombination as the mechanism of telomere formation because in the latter case this flanking DNA would be expected to consist of telomeric repeats. In the three cell lines in which the chromosomal sequences immediately proximal to the construct have been examined, no telomeric repeats were present, and no sequence similarity was identified. Therefore, in these three independent cell lines the telomere-containing con-

struct appears to have integrated randomly, breaking chromosomes and providing telomere function.

The frequency with which this construct forms a new telomere and the simplicity of the basic assay (lack of a discrete terminal restriction fragment) makes it feasible to use this approach to analyze the sequence organization necessary for an introduced telomeric sequence to be efficiently recognized and stabilized by the recipient cell. The minimal lengths of terminal-repeat arrays necessary for the survival of a chromosome and the rate of generation of variability in different cell types can be assayed; the hypothesis that ageing cells have a lower level of telomerase might predict a testable reduction in the frequency of telomere formation by introduced constructs. It is of interest that in mammalian cells a marker gene such as *hisD* can be actively expressed when placed immediately adjacent to the telomeric DNA. This event contrasts with the position effect seen at *S. cerevisiae* telomeres (39).

A high frequency of telomere formation and stabilization by breakage could provide a useful tool for mapping mammalian chromosomes. Such a random approach would constitute a powerful method for the creation of panels of cell lines consisting of sets of nested chromosomal deletions. These panels would be generally useful for the physical mapping of chromosomes; the panels could be used for end-labeling and pulsed-field gel electrophoresis mapping strategies (27, 28) and for the recovery of terminal yeast artificial chromosome clones. Coupled with gene targeting, breaks could be made at defined sites on chromosomes to resolve specific problems of marker order, distance, and orientation. Moreover, if the availability of functioning telomeres can be exploited to produce vectors capable of survival as extrachromosomal linear molecules in mammalian cells, this procedure would represent a step toward the development of mammalian artificial chromosomes.

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