Nucleic Acids Research, 1993, Vol. 21, No. 1 27–36

Telomere directed fragmentation of mammalian chromosomes

Michael A.Barnett, Veronica J.Buckle[1], Edward P.Evans[2], Andrew C.G.Porter, Derek Rout[3], Austin G.Smith[3] and William R.A.Brown*

Biochemistry Department, Oxford University, South Parks Road, Oxford OX1 3QU, [1]Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, [2]MRC Radiobiology Unit, Chilton, Didcot, Oxfordshire, OX11 0RD and [3]AFRC Centre for Genome Research, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JQ, UK

Received October 29, 1992; Revised and Accepted November 27, 1992

ABSTRACT

Cloned human telomeric DNA can integrate into mammalian chromosomes and seed the formation of new telomeres. This process occurs efficiently in three established human cell lines and in a mouse embryonic stem cell line. The newly seeded telomeres appear to be healed by telomerase. The seeding of new telomeres by cloned telomeric DNA is either undetectable or very inefficient in non-tumourigenic mouse or human somatic cell lines. The cytogenetic consequences of the seeding of new telomeres include large chromosome truncations but most of the telomere seeding events occur close to the pre-existing ends of natural chromosomes.

INTRODUCTION

Mammalian artificial chromosomes (MACs) would offer new opportunities for introducing large numbers of genes in a defined sequence environment into experimental animals, agricultural livestock, human somatic cells *in vivo* or mammalian cells in tissue culture (1). In the yeast *Saccharomyces cerevisiae* transformation with telomeric DNA can be used to fragment both natural and artificial chromosomes (2) and has become the basis of a powerful technology for mapping sequences in natural and artificial yeast chromosomes (3, 4). Preliminary evidence suggests that cloned telomeric DNA can also fragment mammalian chromosomes (5). These observations indicate one route to the construction of a MAC. The first step in this involves fragmenting a natural mammalian chromosome with cloned telomeric DNA to produce a mini-chromosome. If such a mini-chromosome were small enough then it might be shuttled into an experimental environment where it could be analysed and manipulated more easily than in a mammalian cell. The nucleus of *S.cerevisiae* would be one such environment. If the mini-chromosome could be re-introduced into a mammalian cell and retain its integrity then it could become the basis of a MAC vector. Central to such a project is the use of cloned telomeric DNA as a reagent for fragmenting mammalian chromosomes.

The work described in this paper demonstrates that cloned human telomeric DNA can efficiently fragment mammalian chromosomes in several mammalian cell types including mouse embryonic stem (ES) cells. We show that when the cloned telomeric DNA seeds the formation of a new telomere it is healed by an enzymatic machinery with the characteristics of telomerase. We have characterized the products of fragmentation in a human cell line and in mouse ES cells by cytogenetic techniques. These include truncated centromere containing versions of natural chromosomes. We have not detected acentric fragmentation products. These results demonstrate that cloned telomeric DNA can be used as a reagent to manipulate the structure of mammalian chromosomes.

MATERIALS AND METHODS

DNA manipulations

Plasmids were constructed by standard procedures from the following fragments. We used the 2.2 kb AccI BamHI fragment of pSV_2neo (6) as the source of the G418 resistance gene in the plasmids pTZsvneoproTEL, pBSsvneoTEL′ and pTZsvneo. We used the 1.8 kb HindIII BamHI fragment from pPGKneoβ (referred to as pDEneo in ref. 7) as the source of the G418 resistance gene in the plasmid pBSPGKneoTEL. In the construction of this plasmid it was assumed that the early region polyadenylation sequence was in the same position with respect to the BamHI site as in pSV_2neo. Subsequently we obtained sequence information about a precursor of pPGKneoβ and realised that the poladenylation sequence is inverted with respect to the BamHI site. Our construct therefore lacks a polyadenylation sequence. The telomeric DNA in pTZsvneoproTEL extended from the PstI site at position 678, 2.4 kb to a Bal31 deletion endpoint within TelSau2.0 (8). The telomeric DNA in pBSsvneoTEL′ (TEL′ is referred to as TELHS in Itzahki *et al.*,

* To whom correspondence should be addressed

in press) and pBSPGKneoTEL extended from the TaqI site at postion 1831 to the deletion end point in the same deleted variant of TelSau2.0.

Extraction of genomic DNA, restriction enzyme digestion, Bal31 digestion, gel electrophoresis and filter hybridization were as described previously (8, 9)

PCR and DNA sequencing

The primers used to amplify the DNA healed onto the linearized pBSsvneoTEL′ were termed 5-amp and C-strand. Their sequences were GACTGAGCTCAGGGGGAATTATCAAGC-TAT and TATAAGCTTCCCTAACCCTGACCCTAACCC respectively. Both were tagged at the 5′ end with a stretch of DNA which was not complimentary to any known sequence in the potential target, which included sites for either SacI (5-amp) or HindIII (C-strand) and which, in the case of C-strand, served to enhance the amplification of the products of the initial round of amplification through subsequent rounds. The PCR reactions were carried out in a total volume of 0.1 mL of 50 mM KCl, 10 mM Tris−HCl pH8.4, 2.0 mM $MgCl_2$, 0.1 mg/mL gelatin, 10^{-7}M 5-amp, 10^{-7}M C-strand, 0.2 mM of each dNTP, 2.5 units Taq polymerase (Cetus) and approximately 0.1 μg of genomic DNA. Annealing was at 66°C for 2 minutes, elongation at 72°C for 2 minutes, and denaturation at 94°C for 2 minutes for a total of 35 cycles. PCR products were partially purified by two cycles of geneclean (Bio101), digested with HindIII and SacI, cloned into pBSKS+ and sequenced by standard double stranded DNA sequencing tehniques using Sequenase II (USB).

Cell culture

The human cell lines HeLa (10), EC27C4 (11), HT1080 (12) and the mouse cell line 10T1/2 (13) were all grown as adherent cells in Dulbecco's modified Eagles Medium supplemented with 10% Foetal Calf Serum, 2 mM L-glutamine and antibiotics (DMEM). The primary human cell line was established as an outgrowth from a portion of a foetal limb and was maintained in supplemented Dulbecco's modified Eagles medium further supplemented with DMEM conditioned by the growth of rat or mouse thymocytes. Cells of this line displayed density dependent inhibition of proliferation and started to senesce after about 25 passages. The mouse ES line, EFC-I, was maintained as described in ref. 14. Cells were transformed with DNA by electroporation using a Bio-Rad gene pulsar typically set to 400V/250 μF and transformants were selected by the growth of the cells in medium containing G418 at 250−300 μg/mL. The transformation efficiencies and protocols varied beween different cell lines and varied between experiments. The frequency of telomere seeding however was consistent for one cell line from experiment to experiment. No systematic differences were observed between the transformation efficiencies observed with pTZsvneoproTEL and pSV_2neo suggesting that the effect of any adjacent telomere upon the expression of the svneo gene was small.

Cytogenetic analysis

Chromosomes were prepared from the HT1080 cells which had been transfected with pTZsvneoproTEL by growing the cells for 12−16 hours in medium supplemented with 100 μg/mL 5-bromo-2-deoxyuridine and then for a further 5−12 hours in medium further supplemented with 10^{-5}M thymidine. Colcemid was added to the culture ten minutes prior to harvesting to a final concentration of 10 μg/mL. Cells were harvested without trypsinization using a solution of 40 mM KCl, 0.5 mM EDTA, 20 mM Hepes pH 7.4, fixed, spread and G banded by standard techniques (15). *In situ* hybridization, detection and replication banding procedures were all performed as previously described (15). The probe used for the *in situ* hybridization was a recombinant of the svneo fragment of pSV_2neo in pTZ18R, referred to as pTZsvneo, labelled with ^3H by nick translation. After the hybridization, exposure and developing of the photographic emulsion the chromosomes were banded with Hoechst 33258 and Giemsa. *In situ* hybridization of the chromosomes isolated from the ES cells was performed after chromosome isolation as described in ref. 16. After the hybridization, exposure and development of the photographic emulsion the chromosomes were banded by trypsin digestion and Giemsa staining.

RESULTS

Functional properties of cloned human telomeric DNA in HeLa cells

We chose to start our investigation of the functional properties of cloned telomeric DNA in the HeLa cell line for two reasons. Firstly, HeLa cells have been shown to contain telomerase activity (17). It seemed likely that this activity would be necessary to heal any newly seeded telomere. Secondly, HeLa cells are aneuploid and contain many rearranged chromosomes. It therefore seemed unlikely that any loss of chromosomal material accompanying a fragmentation event would be fatal to these cells. We constructed the plasmid pTZsvneoproTEL (Figure 1). This plasmid contains a 2.4 kb stretch of human telomeric DNA from the plasmid TelSau2.0 (8), a gene encoding resistance to the antibiotic G418 which is transcriptionally active in human fibroblasts and plasmid vector sequences. The 2.4 kb stretch of human telomeric DNA includes 1.4 kb of human proterminal DNA and 1.0 kb of the $(TTAGGG)_n$ array. The yeast telomeric DNA in TelSau2.0 was deleted by Bal31 digestion prior to plasmid construction. pTZsvneoproTEL was linearized to reveal the human telomeric DNA in its natural orientation at one end of the molecule and introduced into HeLa cells by electroporation. Stably transfected cells were cloned. DNA extracted from the clones was analysed by restriction enzyme digestion, gel electrophoresis and filter hybridization. Digestion with an enzyme cutting at a unique site within pTZsvneoproTEL and hybridization with a probe lying on the telomeric side of the site should give information about the fate of the telomeric DNA in the integrated construct. If the telomeric DNA had seeded the formation of a new telomere then we should expect the filter hybridization analysis to detect a heterogeneous collection of fragments. If the construct had integrated into a chromosome without seeding the

Figure 1. Structure of the constructs used in this work.

formation of a new telomere then we should expect to detect a discretely sized fragment. We analysed 40 stably transfected clones in this way. Figure 2 illustrates the results of a typical set of analysis of 20 clones. The 40 clones contained 44 stable integration events of which 24 were associated with heterogeneously sized fragments (Table 1). These clones contained either one or two copies of the construct. We mapped restriction sites flanking the construct in four clones containing a single integration site and demonstrated that there was a single construct at each and that the integration sites differed from one another. The enzyme used in the experiment illustrated in Figure 2 was HindIII; this cuts at a site 4.7 kb from the boundary with the telomeric DNA in pTZsvneoproTEL. If the heterogeneously sized fragments do correspond to newly seeded telomeres then the results of the experiment illustrated in Figure 2 suggest that they range between 1 kb and 20 kb in size. We wanted to confirm that the construct in this sort of clone lay at the end of a chromosomal DNA molecule and so we digested DNA extracted from three such clones with the exonucease Bal31. The digests were sampled periodically and analysed by restriction enzyme digestion, gel elecrophoresis and filter hybridization as described above. In each of these three clones the cognate restriction fragments were sensitive to the action of the Bal31 (Figure 3a–c). In order to check the specificity of our approach we also analysed a clone which contained a construct which had stably integrated into a HeLa cell chromosome without appearing to seed the formation of a new telomere. As anticipated the cognate fragments in this clone were not detectably sensitive to the action of Bal31 (Figure 3d). In the experiment illustrated in Figure 3 the DNA was digested with XbaI after Bal31 digestion. XbaI does not cut within the integrated pTZneoproTEL but was used in this experiment becase it allowed us to use a hybridization probe specific for the neo gene which produced lower non-specific background after filter hybridization than the vector probe used in Figure 2.

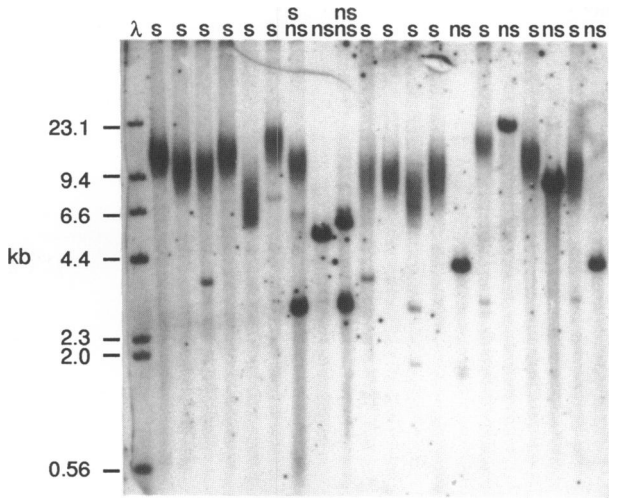


Figure 2. Detection of Telomere seeding activity by gel electrophoresis and filter hybridization. DNA extracted from clones of HeLa cells that had been stably transfected with pTZsvneoproTEL was digested with HindIII, electrophoresed on a 0.8% agarose gel, filter transfered and hybridized to ^{32}P-labelled pTZ18R. The first track includes an end labelled HindIII digest of phage λ DNA and is labelled λ. Subsequent tracks are labelled s or ns to indicate whether the pTZsvneoproTEL has seeded a new telomere.

We tried to confirm the chromosomal location of the integrated construct by fluorescent *in situ* hybridization but were unable routinely to detect the short stretch of heterologous DNA in this construct using this technique.

We were interested to know whether the ability to seed new telomeres in this way required the precence of telomeric DNA

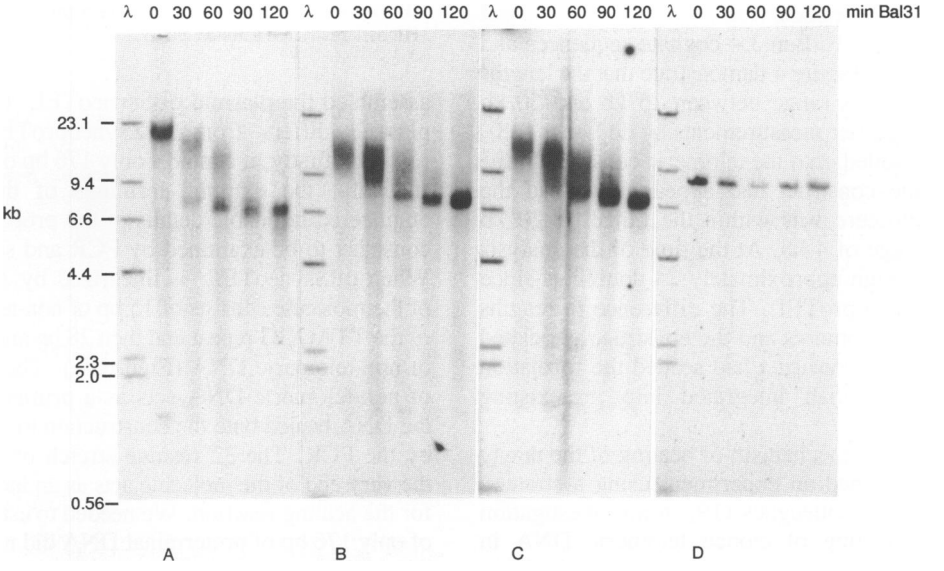


Figure 3. Bal31 sensitivity of newly seeded telomeres. DNA extracted from each of four HeLa derived clones which had been stably transfected with pTZsvneoproTEL was incubated for the indicated time with Bal31. The reaction was terminated with EGTA and phenol. DNA was extracted and further digested with XbaI. Digests were analysed by gel electrophoresis and filter hybridization with a 0.8 kb PvuII fragment from the the neomycin resistance gene in pSV$_2$neo. One track from each panel includes an end labelled HindIII digest of phage λ DNA. Panels A–C correspond to clones where the construct has seeded a new telomere, panel D corresponds to a clone where no detectable seeding has occurred. The clones used in this experiment do not correspond to any of those used in the experiment illustrated in Figure 2.

Table 1. Frequency of telomere seeding in mouse and human cell lines

Cell line	Origin	Construct	Clones	Events	Seeded	Not-Seeded	Fraction
HeLa	Human	pTZsvneoproTEL	40	44	24	20	0.55
HeLa	Human	pSV_2neo	20	27	0	27	<0.04
HeLa	Human	pBSsvneoTEL'(Asp718I†)	43	45	20	25	0.45
HeLa	Human	pBSsvneoTEL'(ApaI†)	39	47	17	30	0.36
EC27C4	Human	pTZsvneoproTEL	39	54	34	20	0.63
HT1080	Human	pTZsvneoproTEL	22	27	13	14	0.55
1°-FT	Human	pTZsvneoproTEL	40	54	0	54	<0.02
ES EFC	Mouse	pBSPGKneoTEL	29	30	11	19	0.36
10T1/2	Mouse	pTZsvneoproTEL	38	53	1	52	0.02

†Indicates the enzyme used to linearize pBSsvneoTEL'.

in the construct and so we transfected HeLa cells with linearized pSV_2neo. We analysed 20 clones in the way described above. None of the 27 different integration events detectable in these clones appeared to be associated with a new telomere (data not shown).

The fate of cloned telomeric DNA upon introduction into HeLa cells

The results described in the previous section suggested that the cloned telomeric DNA in the construct pTZsvneoproTEL had integrated into the HeLa cell chromosomes and seeded the formation of a new telomere. The results were also consistent with the possibility that the construct had integrated into pre-existing telomeres. This seemed less likely when we compared the average lengths of the construct associated telomeres and of the endogenous HeLa cell telomeres. In order to make this comparison we measured the average lengths of the endogenous telomeres by restriction enzyme digestion, pulsed field gel electrophoresis and filter hybridization with the TelBam3.4 probe (8) (Figure 4). In this experiment we digested the DNA with BamHI prior to gel electrophoresis and filter hybridization. BamHI cuts 3.4 kb from the boundary between the telomeric and the proterminal DNA in the TelBam3.4 cognate sequences and thus the results illustrated in Figure 4 demonstrate that the lengths of the endogenous telomeres range between 15 kb and 50 kb which is consistent with earlier measurements by de Lange (18). The length of sequence added onto the telomeric construct in the 24 clones in which the construct had appeared to seed the formation of a new telomere was within the range 0−20 kb (Figure 2) with an average of 4 kb. At the time of the analysis the clones had been through approximately 24 doublings since transfection with pTZsvneoproTEL. The difference in lengths between the endogenous telomeres and the construct-associated telomeres suggests that the construct had seeded the formation of new telomeres rather than integrated into pre-existing telomeres.

In order to investigate the mechanism of healing of the newly seeded telomeres we designed an experiment using a strategy established by Murray and colleagues (19) in an investigation of the mechanism of healing of cloned telomeric DNA in *S.cerevisiae*. These workers introduced a construct containing cloned telomeric DNA with a non-telomeric polylinker extension into yeast and observed that the construct was healed with telomeric DNA but retained some or all of the polylinker sequences. They concluded that the construct was healed by a mechanism which did not involve sequence conversion or recombination and was likely to involve telomerase. We therefore

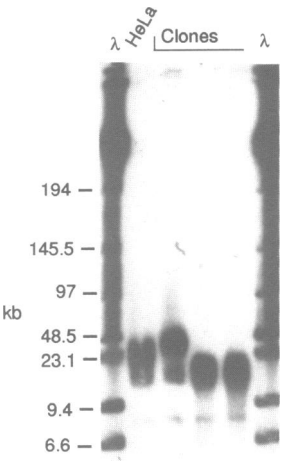


Figure 4. Lengths of endogenous telomeres in HeLa cells and in three transfected clones. DNA from the HeLa cell line or from each of three stably transfected clones was restricted with BamHI, size fractionated by pulsed field gel electrophoresis and analyzed by filter hybridization with the TelBam3.4 probe (Brown *et al.*, 1991). Markers were a mixture of multimers of λ DNA and HindIII restricted lambda DNA.

assembled the plasmid pBSsvneoTEL' (Figures 1 and 5). This plasmid differs from pTZsvneoproTEL in two significant respects: firstly, it contains only 176 bp of proterminal DNA and secondly, the extreme terminus of the molecule has been engineered to enable details of the processing of the end of the construct to be examined by PCR and sequencing (Figure 5a). When pBSsvneoTEL' is linearized by ApaI the telomeric end of the molecule consists of 15 bp of non-telomeric DNA, 2 copies of the TTAGGG repeat and then 28 bp and 4 unpaired 3' residues of non-telomeric DNA (Figure 5a). The recessed 15 bp stretch of non-telomeric DNA acts as a primer binding site to enable the DNA healed onto the construction to be specifically amplified by the PCR. The 32 residue stretch of non-telomeric DNA at the very end of the molecule acts as an indicator of the specificity for the healing reaction. We needed to establish that the presence of only 176 bp of proterminal DNA did not impair the functional properties of the TTAGGG array before we used this construct to examine the mechanism of healing of the newly seeded telomeres. There is a unique Asp718I site between the terminal 32 residue stretch of non-telomeric DNA and the adjacent TTAGGG sequence and so in initial experiments we linearized pBSsvneoTEL' with Asp718I, transfected the plasmid into HeLa cells by electroporation, isolated clones and analyzed the extracted

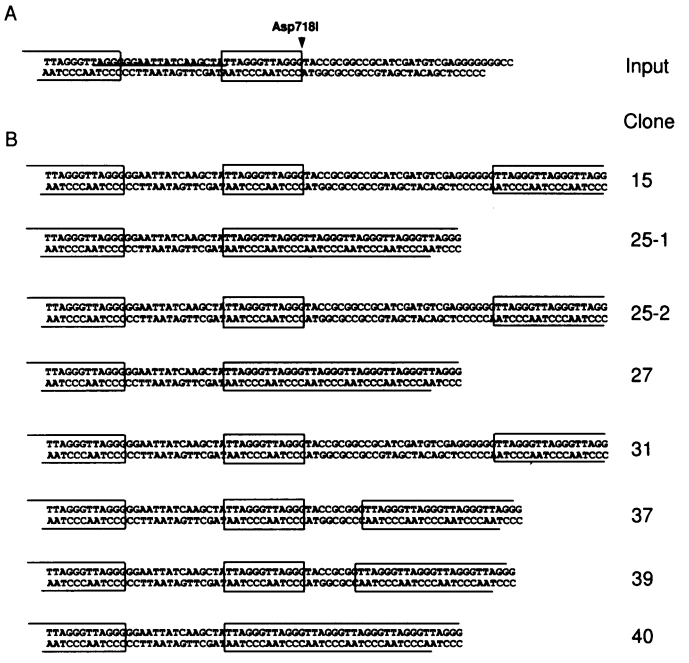


Figure 5. Specificity of telomere healing in HeLa cells. A. The sequence corresponds to the telomeric terminus of the ApaI linearized pBSsvneoTEL′. The region underlined is the cognate sequence of the specific primer 5-amp used to amplify DNA healed onto the construct. The boxed sequences correspond to TTAGGG repeats. The Asp718I site used in the preliminary experiment is indicated. B. Sequences of DNA at the termini of healed ApaI cut pBSsvneoTEL′. Two sequences were detected in the PCR products if clone 25 and these are indicated as 25−1 and 25−2.

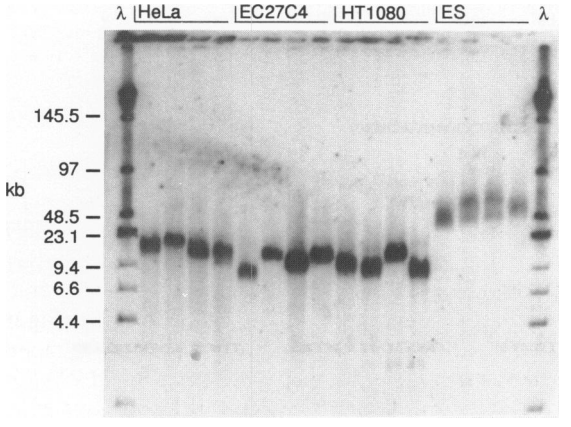


Figure 6. Lengths of DNA healed onto the newly seeded telomeres in different cell lines. DNA isolated from four clones derived by transfection of the indicated lines with either pTZsvneoproTEL (HeLa, EC27C4 and HT1080) or pBSPGKneoTEL (ES) was digested with either XbaI (HeLa, EC27C4 and HT1080) or HindIII (ES), size fractionated by pulsed field gel electrophoresis and analyzed by filter hybridization with the 0.8 kb PvuII fragment from the neomycin resistance gene of pSV_2neo. Markers were a mixture of multimers of λ DNA and HindIII restricted lambda DNA.

DNA using the biochemical approach established above. In 20 of the 43 stably transfected clones that we analysed the linearized construct seeded the formation of a new telomere. We therefore concluded that, as anticipated, the proterminal sequences present

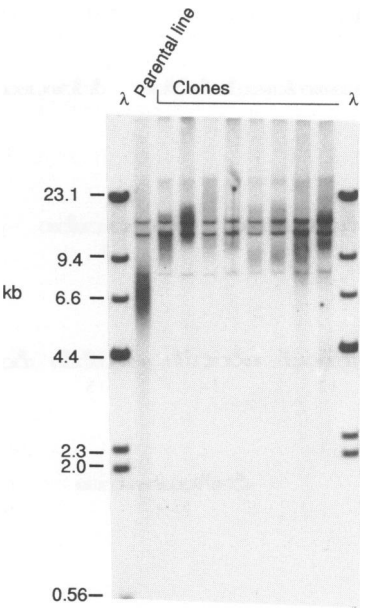
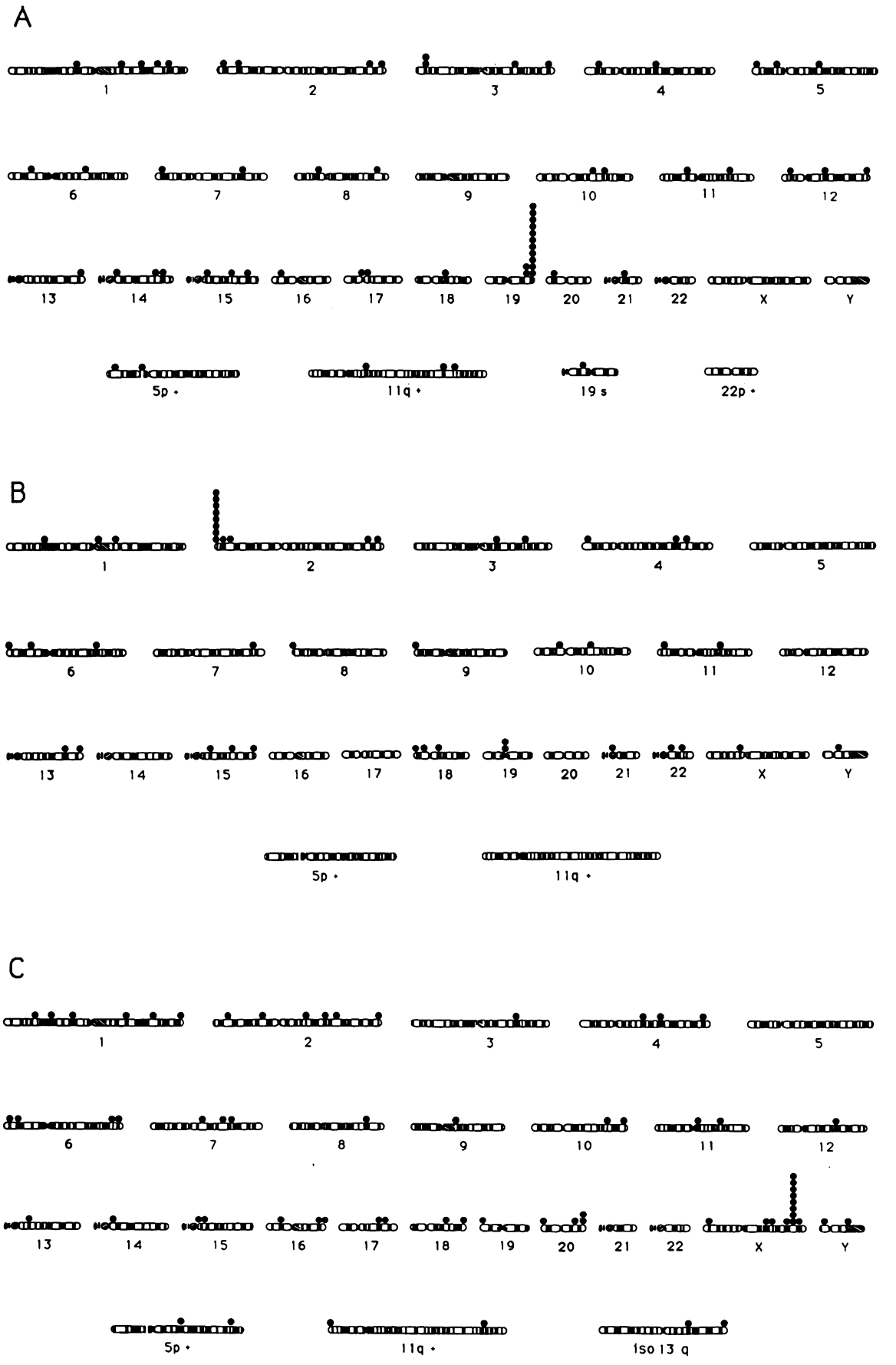


Figure 7. Telomere growth during cloning and proliferation of primary human fibroblasts. Primary human fibroblasts of the 1°FT line were transfected with linearized pTZsvneoproTEL and stably transformed cells were cloned. DNA was extracted from the clones, digested with BamHI and analyzed by gel electrophoresis and filter hybridization with the TelBam3.4 probe.

in pTZsvneoproTEL, but absent from pBSsvneoTEL′, were without significance for this aspect of telomere function. In five of the clones in which the construct had seeded the formation of a new telomere we were able to generate a PCR product which hybridized to ^{32}P-$(TTAGGG)_4$ when we amplified with primers complimentary to the G rich strand of the human telomeric repeat and to the 15 basepair recessed primer binding site. We cloned and sequenced one of the PCR products and demonstrated that the construct had been healed with $(TTAGGG)_n$ on or within the $(TTAGGG)_2$ sequence (not shown). We interpreted the failure to generate a PCR product in other fifteen clones where the construct had seeded the formation of a new telomere as indicating that the construct had been healed behind the boundary of the $(TTAGGG)_2$ repeat and thus had lost the sequence complimentary to the specific primer. In the next experiment we linearized the construction with ApaI, electroporated HeLa cells and analysed 39 stably tranfected clones. In 17 of these the construct was associated with a newly seeded telomere suggesting that the non-telomeric DNA extension did not significantly impair the functional properties of the cloned telomeric DNA. Seven of these clones yielded a PCR product. We cloned and sequenced two or three copies of the individual products. The results (Figure 5b) demonstrate that the machinery responsible for healing the end of our construct does not require $(TTAGGG)_n$ to be present at the very end of the molecule. This observation strongly suggests that the construct has been healed by a mechanism that does not involve recombination or gene conversion. It seems probable that our construct has been healed by telomerase. These results also provide further evidence against the notion that in these clones the construct has simply integrated into a pre-existing telomere.

The newly seeded telomeres were smaller than the endogenous telomeres when we examined them first. We were therefore


A
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
5p -
11q -
19 s
22p -
B
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
5p -
11q -
C
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
5p -
11q -
Iso 13 q

interested to know whether the lengths of these two classes of telomere changed on prolonged culture and, if so, at what rate. We analysed the lengths of the newly seeded telomeres and of the endogenous telomeres at intervals corresponding to 60 population doublings for a total of 300 population doublings. The average lengths of the newly seeded telomeres progressively approached those of the endogenous telomeres and became more heterogeneous. Assuming a uniform growth rate for all the cells in the culture, the maximum rate of telomere growth in the newly seeded telomere was approximately 130 bp per cell doubling (data not shown). We did not detect the appearance of any discretely sized fragments in the course of this analysis indicating that, once they are formed, the telomeres are stable.

In approximately half of the clones which had been stably transfected with either of the telomeric constructs, the construct had appeared to fail to seed the formation of a new telomere. We were also interested to know the fate of the cloned telomeric DNA in these clones. In order to address this point we made use of a unique EcoNI site present in the proterminal DNA of pTZsvneoproTEL. Restriction analysis demonstrated that this site was undetectable in 13 out of 15 integration sites associated with the failure to seed a new telomere (data not shown). The site was detectable in each of the 22 sites in the 22 clones in which pTZsvneoproTEL had seeded the formation of a new telomere. These results suggest that the failure of the construct to seed a new telomere is associated with the loss of telomeric DNA from the construct. We analysed the structure of the integration site in one such clone at intervals of 60 population doublings for a total of 300 population doublings and, as anticipated, failed to detect any evidence of resolution of the construct into a telomere. Similarly, restriction site analysis of the DNA flanking the integration sites in one clone in which the construct existed in both telomeric and non-telomeric locations demonstated two independent sites of integration. These results thus suggest that a 1kb interstitial stretch of $(TTAGGG)_n$ is unstable and that chromosome breakage and telomere seeding occurs simultaneously or very soon after integration of the construct into the chromosome.

Telomere seeding in other mammalian cell types

The results of the previous section demonstrate that cloned telomeric DNA can seed the formation of new telomeres efficiently upon integration into HeLa cell chromosomes. We wanted to examine the functionality of cloned telomeric DNA in a variety of other mammalian cell types for three reasons. First of all we wanted to determine the cytogenetic consequences of telomere seeding and if possible to demonstrate directly that cloned telomeric DNA could fragment mammalian chromosomes. We therefore needed to detect telomere directed chromosome fragmentation in a mammalian cell type with a easily defined set of chromosomes. Secondly, we want to be able to fragment chromosomes in the mouse germ line. We therefore needed to demonstrate telomere directed chromosome fragmentation in mouse embryonic stem (ES) cells. Thirdly, we were interested to know how different cell types process cloned telomeric DNA.

We started our analysis with a human embryonic fibroblast line, 1°-FT, established in this laboratory. We chose to work with a primary fibroblast line in the first experiments because cytogenetic analysis of such lines is relatively straightforward. We transfected cells from the second passage of this line with linearized pTZsvneoproTEL and analysed 40 stably transfected clones by the molecular techniques established above. Fifty-four different integration events were detectable but in none of these had the construct seeded the formation of a new telomere. We therefore analysed the ability of cloned telomeric DNA to seed the formation of new telomeres in two established human cell lines; the teratocarcinoma EC27C4 and the fibrosarcoma HT1080 both of which have been reported to contain a recognizable set of human chromosomes. We transfected cells of each line with the linearized pTZsvneoproTEL and analyzed stably transfected clones. The construct efficiently seeded the formation of new telomeres in both lines. (Table 1). We next analyzed the ability of the construct pBSPGKneoTEL (Figure 1) to seed the formation of new telomeres in the ES line EFC. We chose to use a construct which included a G418 resistance gene driven by the promoter of the mouse phosphoglycerate kinase gene because the sv40 early region is poorly expressed in ES cells. We analysed 29 stably transfected ES clones and detected 30 independent integration events of which 11 were associated with the formation of a new telomere. During the initial stages of this analysis we noted that the newly seeded telomeres were associated with a much longer stretch of telomeric DNA in the ES cells than in any of the other cell types (Figure 6). Two practical consequences of this difference are that reliable detection of telomere seeding in ES cells requires the use of pulsed field gel electrophoresis and that the ES cell DNA is best extracted in agarose plugs in order to retain its integrity prior to analysis. The observation that the construct is often associated with a long stretch of telomeric DNA is also consistent with the possibility that in some of these cell lines it has simply integrated into pre-existing telomeres. However we assume that ES cells are not qualitatively different from the other cells in which telomere seeding is observed and that the our construct has in fact seeded the formation of new telomeres in a majority of the ES cell clones where it has been healed with a large tract of DNA. Cytogenetical analyses described below support this view. We were curious to know whether the ability of the ES cells to heal the cloned telomeric DNA with a long stretch of $(TTAGGG)_2$ was a consequence of their being of mouse or of germ line origin. We therefore transfected mouse 10T1/2 cells with linearized pTZsvneoproTEL. These are somatic cells but unlike many established mouse somatic cells share with ES cells endogenous telomeres which range in length between 25 and 75 kb (not shown). We analyzed 38 stably transfected clones containing a total of 53 different integration events and

Figure 8. *In situ* hybridization analysis of HT1080 clones stably transfected with telomeric DNA. Distribution of silver grains scored over replication banded chromsomes of HT1080 cells stably transfected with pTZsvneoproTEL and probed with ^{3}H-labelled pTZsvneo. A. Clone 17.2 has a specific signal at 19qter, scored in 25 cells. B. Clone 16.1 has a specific signal at 2pter scored in 20 cells. C. Clone 6.3 has a specific signal at Xq26 scored in 17 cells. The panels in the figure also include ideogramatic representations of re-arranged chromosomes present in each of the the three karyotypes. The prescence of these chromosomes reflect either core abnormalities in the karyotype of the parental HT1080 line or re-arrangements which have occurred subsequently to tranfection but are unrelated to the precence of the cloned telomeric DNA in the line. The karyotype of the parental HT1080 line is polyclonal with diploid and tetraploid forms of each clone. Core abnormalities are 5p+ and 11q+ with an iso13q present in a minority of cells. The 17.2 line has a karyotype 46 XY, 5p+, 11q+, 19s, 22p+. The 16.1 line has the karyotype 46 XY, 5p+, 11q+. The 6.3 line has the karyotype 90 XXYY, 5p+, 5p+, 11q+, 11q+, iso13q, iso13q.

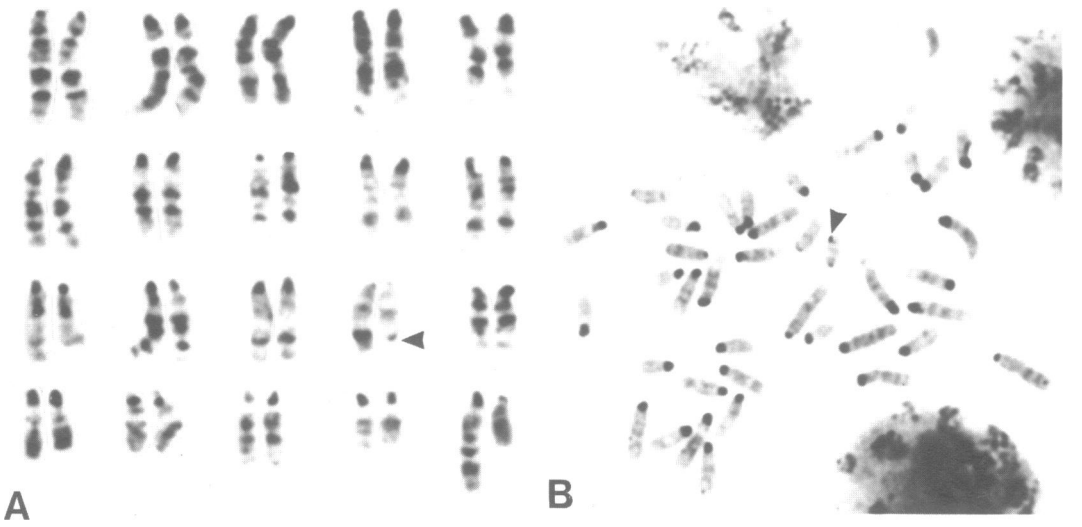


Figure 9. Mouse embryonic stem cell chromosomes from a cell line with a newly seeded telomere and a truncated chromosome 14. A. G-banded karyotype of ES cell clone Tel 2 showing truncated chromosome 14 (arrowhead). B. *In situ* hybridization of ^{3}H-labelled pTZsvneo to ES cell clone Tel 2 chromosomes showing localization of a silver grain to the truncation site on chromosome 14 (arrowhead). We analyzed a total of 100 cells in this way and located 225 grains over the chromosomes. Twenty six of these were on the deleted chromosome 14 and 25 at the truncation site. The truncated chromosome 14 comprises approximately 3.2% of the karyotype and thus there is a significant excess of hybridization to the truncation breakpoint compared to random.

were able to detect only one in which the construct may have seeded the formation of a new telomere. This 'clone' either contained two integration events or was a mixture of two clones and analysis of the structure of the integrated construct was pursued unsuccesfully. It therefore remains unknown whether the ability of the ES cells to heal the cloned telomeric DNA with a long stretch of telomeric DNA reflects their being of mouse or of germ line origin. The results of the analysis of the 10T1/2 cells however provide futher evidence that telomere seeding by cloned telomeric DNA is inefficient in non-transformed somatic cells.

Telomere seeding by cloned telomeric DNA appeared to be efficient in the established human cell lines; HeLa, HT 1080 and EC27C4 and in ES cells, to be very inefficient in the 10T1/2 cells and undetectable in the human primary fibroblasts. We wondered whether the failure to detect telomere seeding in the primary fibroblasts might reflect a lack of telomerase in these cells. In order to investigate this possibility we compared the lengths of the endogenous telomeres in the parental primary fibroblast line and in eight of the stably transfected clones isolated after about 20 population doublings. We digested DNA from each of these sources with BamHI and analysed the digests after gel electrophoresis and filter transfer with the TelBam 3.4 probe (Figure 7). This probe recognizes about 12 different telomeric loci and a single complex non-telomeric locus (8). The telomeric loci correspond to the heterogeneous sized fragments which in the parental line are of average of 6 kb in size. In the stably transfected clones the heterogeneously sized fragments are longer than in the parental clones and range in size between 9.4 kb (clone 5) and 15.0 kb (clone 2). These results suggest that the telomeres in these clones have grown during the course of the experiments and that the clones contain functional telomerase.

Cytogenetic consequences of telomere seeding

The demonstration that cloned telomeric DNA could seed the formation of a new telomere upon introduction into cells with a recognizable set of chromosomes suggested that the cytogenetic consequences of the seeding of a new telomere were amenable to analysis. We were unable to use fluorescent techniques to detect our short constructs in metaphase chromosomes. We therefore hybridized ^{3}H-labelled pTZsvneo to metaphase chromosomes of two clones of HT1080 cells, clones 17.2 and 16.1, where the molecular analysis described in the previous section demonstrated that the pTZsvneoproTEL had seeded the formation of a new telomere. The results of the cytogenetic analysis indicated that in each of these two clones the construct lay at the end of a recognizable chromosome. In clone 17.2 it lay at the end of the long arm of chromosome 19 (Figure 8a) and in clone 16.1 it was located at the end of chromosome 2 short arm (Figure 8b). We could however detect no chromosome truncation in either of these clones. In light of the biochemical evidence presented above we interpret this result to suggest that in each of these clones the cloned telomeric DNA has integrated close to a pre-existing natural telomere. It was particularly important to confirm that the cloned telomeric DNA could seed the formation of a new telomere in ES cells and so we examined six clones where the molecular analysis indicated that the pBSPGKneoTEL had stably integrated into the ES cell genome. There was no evidence of chromosome truncation in four of these clones however two of the clones contained cytogenetically distinct truncated versions of chromosome 14 and were hemizygous for sequences distal of the truncation breakpoint. *In situ* hybridization analysis of one of these clones demonstrated that the pPGKneoTEL lay at the breakpoint of the truncated copy of chromosome 14 (Figure 9). The simplest interpretation of all of these data is that when cloned telomeric DNA integrates into mammalian chromosomes and seeds the formation of a new telomere it tends to do so near the end of a pre-existing chromosome. It can however occasionally integrate into the bulk of a chromosome and cause a cytogenetically detectable truncation.

We were also interested to know whether there were any cytogenetic consequences of a construct containing cloned telomeric DNA integrating into a chromosome and failing to seed the formation of a new telomere. We analysed one HT1080 clone,

clone 6.3, where the molecular analysis demonstrated that pTZsvneoproTEL had integrated but failed to seed the formation of a new telomere (Figure 8c). In clone 6.3 the construct had integrated into Xq26 without being associated with a cytogenetically detectable rearrangement.

DISCUSSION

We initiated this project in order to determine whether we could use cloned telomeric DNA to fragment mammalian chromosomes. The combination of molecular and cytogenetic analyses described here, together with the results of Itzahki and colleagues (in press) showing truncation of human chromosome 1 by targeted telomere directed breakage, demonstrate that we can. This process occurs efficiently in the established cell lines HeLa, EC27C4 and HT1080 and in the mouse embryonic stem cell line EFC but is either inefficient or undetectable in mouse 10T1/2 cells or in human primary fibroblasts. The cytogenetic consequences of the seeding of a new telomere include the generation of large chromosome truncations but most of the integration events associated with the formation of newly seeded telomeres appear to occur close to the pre-existing ends of natural chromosomes and to generate, consequently, truncations which are too small to detect by cytogenetic techniques. These observations raise two questions. Why does cloned telomeric DNA not detectably seed the formation of new telomeres in human primary fibroblasts and why do most of the seeding events appear to occur close to the pre-existing ends of chromosomes?

We analysed 54 sites where pTZsvneoproTEL had integrated into the genome of a human primary fibroblast line but at none of these had a new telomere been seeded. One possible explanation for a failure to detect telomere seeding might be an absence of telomerase from the primary fibroblast line (20). If this was the case then the cloned telomeric DNA could have seeded the formation of new telomeres but the telomeres would have progressively shorted during culture as a result of exonuclease action and a failure to replicate the 5′ end of the telomeric DNA. If the newly seeded telomere had shortened until it was too small to function then the remnants of the construction would either have been lost as a result of further sequence degradation or would have become the junction of a fusion with another chromosome. If the selectable marker gene in the construct had been destroyed then the clone, grown in the presence of the antibiotic G418 would have died. If the telomere had failed and the construction had fused with another chromosome then the construct would have appeared to have failed to seed the formation of a new telomere. The observation that the endogenous telomeres in the stably transfected clones were longer than those in the parental line however argues against telomerase deficiency being the basis of the correct explanation of our results. Chromosome structure is more stable in primary fibroblasts than in transformed cells and it is possible that the failure to detect telomere seeding by transfected telomeric DNA reflects active repair and recombination pathways which tend to integrate sequences into the genomic DNA of these cells in such a way as to prevent chromosome breakage. Our experiment however does not allow an unambiguous explanation of our results; it is, for example, possible that telomerase was not present in the primary fibroblasts at the time of transfection and that any newly seeded telomeres were lost prior to expression of any telomerase activity. Alternatively we may have selected for those clones with long telomeres. Understanding chromosome breakage, telomere seeding and the role of telomerase will require reconstitution of the events *in vitro* from defined components.

It is striking that most of the seeding events that we detected appear to occur close to the pre-existing ends of the chromosomes. It is unlikely that this will prevent cloned telomeric DNA being used to systematically fragment mammalian chromosomes. In experiments described elsewhere we have truncated human chromosome 1 at band 1p35 by targeting telomeric DNA to the 6−16 locus (Itzhaki *et al.* in press). Nevertheless more work will be needed to establish how reliably we can use sequence targeting and telomere directed chromosome fragmentation to systematically fragment mammalian chromosomes. It seems particularly important that this work be carried out in ES cells where, suprisingly, two of the breaks that we detected were on chromosome 14. It might be thought that that our failure to detect a set of apparently random chromosome truncations was caused by the reduced viability of cells hemizygous for large chromosomal regions. This explanation is unsatisfactory for two reasons. Firstly cells with large truncations of chromosomal material induced by therapeutic irradiation are viable *in vivo* for many years (21). Similarly mice hemizygous for large chromosomal regions can be viable (Cattanach *et al.*, in press). Secondly the HT1080 karyotype appears variable and changes in ploidy which might compensate for the genetic consequences of any truncation should occur readily. We speculate that the non-random pattern of integration of the functioning telomeric DNA reflects an interaction between the cloned telomeric DNA in the construct and telomere binding proteins located at the nuclear periphery prior to the integration of the construct into the chromosome. Cytogenetic analysis also failed to detect large acentric fragments of chromosomal DNA in the clones containing newly seeded telomeres. This may reflect our analysis of relatively few clones by cytogenetic techniques, the fact that most of the breaks generate fragments too small to detect or the mitotic instability of such fragments.

Despite these limitations in our understanding of the molecular events which occur when cloned telomeric DNA breaks a chromosome and seeds the formation of a new telomere it seems reasonable to conclude that we can use cloned telomeric DNA as a reagent for both structural and functional studies of mammalian chromosomes in a variety of transformed cell lines and in the mouse germ line. Furthermore, the observation that cloned telomeric DNA can break chromosomes in mammalian cells which readily integrate non-homologous DNA into chromosomes suggests that telomeric DNA might be used to manipulate chromosome structure in a wide variety of plants and animals.

ACKNOWLEDGEMENTS

We thank Chris Tyler-Smith and Karen Brown for their comments upon a draft manuscript. This work was supported by the Cancer Research Campaign and by the Medical Research Council as part of the UK Human Genome Mapping Programme.

REFERENCES

1. Brown,W.R.A. (1992) *Curr. Opin. Genet. Dev.* **2**, 479−486.
2. Surosky,R.T. and Tye,B.-K. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2106−2110.
3. Vollrath,D., Davis,R. W., Connelly,C. and Hieter,P. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6027−6031.
4. Pavan,W.J., Hieter,P. and Reeves,R.H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1300−1304.

5. Farr,C., Fantes,J., Goodfellow,P. and Cooke,H.J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7006–7010.
6. Mulligan,R.C. and Berg,P. (1980) *Science* **209**, 1422–1428.
7. Rathjen,P.D., Toth,S., Willis,A., Heath,J.K. and Smith,A.G. (1990) *Cell* **62**, 1105–1114.
8. Brown,W.R.A., MacKinnon,P.J., Villasante,A., Spurr,N., Buckle,V.J. and Dobson,M.J. (1990) *Cell* **63**, 119–132.
9. Brown,W.R.A. (1988) *EMBO J.* **7**, 2377–2385.
10. Scherer,W.F., Syverton,J.T. and Gey,G.O. (1953) *J. Exp. Med.* **97**, 695–709.
11. Pera,M.F., Cooper,S., Mills,J. and Parrington,J.M. (1989) *Differentiation* **42**, 10–23.
12. Rasheed,S., Nelson-Rees,W.A., Toth,E.M., Arnstein,P. and Gardner,M.B. (1974) *Cancer* **33**, 1027–1033.
13. Reznikoff,C.A., Brankow,D.W. and Heidelburger,C. (1973) *Cancer Res.* **33**, 3231–3238.
14. Nichols,J., Evans,E.P. and Smith,A.G. (1990) *Development* **110**, 1341–1348.
15. Buckle,V.J. and Craig,I.W. (1986) in Davies,K.E. (ed.), *Human Genetic Diseases*. IRL Press, Oxford.
16. Hogan,B.L.M., Costatini,F. and Lacy,E. (1980) *Manipulating the Mouse Embryo; A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17. Morin,G.B. (1991) *Cell* **59**, 521–529.
18. De Lange,T., Shiue,L., Myers,R.M., Cox,D.R., Naylor,S.L., Killery,A.M. and Varmus,H.E. (1990) *Mol. Cell. Biol.* **10**, 518–527.
19. Murray,A.W., Claus,T.E. and Szostak,J.W. (1988) *Mol. Cell. Biol.* **8**, 4642–4650.
20. Counter,C.M., Avillon,A.A., LeFeuvre,C.E., Stewart,N.G., Grieder,C.W., Harley,C.B. and Baccheti,S. (1992) *EMBO J.* **11**, 1921–1929.
21. Adolph,S., Bartram,C.R. and Hameister,H. (1987) *Cytogenet. Cell Genet.* **44**, 65–68.