Telomere elongation involves intra-molecular DNA replication in cells utilizing alternative lengthening of telomeres

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Alternative lengthening of telomeres (ALT) is a telomere length maintenance mechanism based on recombination, where telomeres use other telomeric DNA as a template for DNA synthesis. About 10% of all human tumors depend on ALT for their continued growth, and understanding its molecular details is critically important for the development of cancer treatments that target this mechanism. We have previously shown that telomeres of ALT-positive human cells can become lengthened via inter-telomeric copying, i.e. by copying the telomere of another chromosome. The possibility that such telomeres could elongate by using other sources of telomeric DNA as copy templates has not been investigated previously. In this study, we have determined whether a telomere can become lengthened by copying its own sequences, without the need for using another telomere as a copy template. To test this, we transduced an ALT cell line with a telomere-targeting construct and obtained clones with a single tagged telomere. We showed that the telomere tag can be amplified without the involvement of other telomeres, indicating that telomere elongation can also occur by intra-telomeric DNA copying. This is the first direct evidence that the ALT mechanism involves more than one method of telomere elongation.

INTRODUCTION

Alternative lengthening of telomeres (ALT) is a non-telomerase-based mechanism activated by a substantial minority of immortal cell lines and tumor types to counteract the telomere erosion due to successive cycles of cell division. The hallmarks of ALT in human cells include the presence of ALT-associated promyelocytic leukemia (PML) bodies (APBs), i.e. nuclear aggregates where telomeric DNA colocalizes with PML protein (1), and the presence of telomeres that are highly heterogeneous in length, ranging from very short to >50 kb (2). The telomere length dynamics of telomerase-negative immortalized cells suggested that this mechanism is based on recombination: single telomeres undergo dramatic length fluctuations during cycles of cell division, with rapid shortening and rapid lengthening events (3,4). In addition, ALT cells are able to elongate their telomeres using telomeric sequences from other chromosomes

as a template for new DNA synthesis (5), a process that can occur anywhere within the length of the telomere (6).

Potentially, other sources of telomeric DNA could be used as templates for telomere elongation. Telomeres are organized in a t-loop structure (7): the single-stranded overhang is able to fold back, invade the double-stranded telomeric DNA tract and anneal to the complementary strand. It has been suggested that t-loop structures may prime telomere elongation (7), but this hypothesis has not been tested previously. ALT cells have increased rates of telomeric exchange events (8,9) that are usually referred to as telomeric sister chromatid exchanges (t-SCE). It has been suggested that these exchanges may be unequal, leading to elongation of one telomere and shortening of the other (10), but no experimental evidence to support this hypothesis is yet available. The other potential source of $(TTAGGG)_n$ template sequences is extrachromosomal telomeric repeat (ECTR) DNA: this is abundant in ALT cells, is

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organized in circular, linear and t-loop forms (11–14) and is at least partly contained within APBs (15). The linear telomeric DNA associated with APBs is unlikely to be a major source of templates for replication, however, because it is predominantly short (15) and would not account for the rapid telomere elongation events observed in ALT cells. It remains unclear whether circular or t-loop-structured ECTR DNA can be used as a copy template by telomeres in ALT cells, e.g. by rolling circle replication.

In this study, we show that a plasmid tag integrated within a single telomere of an ALT cell line can be amplified without the involvement of other telomeres, indicating that telomere elongation can also occur by intra-telomeric DNA copying. We therefore report here for the first time that intra-telomeric duplication of telomeric sequences occurs in ALT cells.

RESULTS

Assay for intra-telomeric sequence duplication

We constructed an intra-telomeric duplication reporter plasmid, referred to as pHRT. When the plasmid is linearized (Fig. 1), the coding sequence of the far-red fluorescent protein HcRed (RFP) is located upstream of the cytomegalovirus (CMV) immediate-early promoter/enhancer (referred to hereafter as the CMV promoter). The splice acceptor and donor sites from the human triose phosphate isomerase (TPI) intron 6 boundaries are situated 5′ to the RFP reporter and 3′ to the CMV promoter sequences, respectively. To obtain telomeric targeting, these sequences are flanked by 800 bp of telomeric sequences. An antibiotic resistance gene, hygromycin phosphotransferase (HPT), was also present to allow selection of cells with integrated plasmid DNA in media containing hygromycin B.

If the pHRT construct integrates at a telomere as a single copy, the CMV promoter will be unable to drive expression as it is located downstream of the RFP sequence. If, however, the construct is duplicated within the same telomere (Fig. 1), one of the CMV promoters will now be upstream of an RFP open reading frame, separated by a splice donor site, a tract of telomeric repeats of variable length and a splice acceptor site. After transcription, splicing of the intervening $(TTAGGG)_n$ tract will be directed by the splice donor and acceptor sites, RFP will be translated from the mature mRNA, and cells will fluoresce.

A recent study demonstrated that telomere sequences can be transcribed from subtelomeric promoters toward the chromosome terminus (16). To prevent expression of the RFP reporter as a consequence of telomeric repeat-containing RNA (TERRA) transcription, we introduced the telomere-targeting construct on the C-rich strand of the telomeres. Hence, any transcription originating from subtelomeric promoters and encompassing the plasmid sequences would result in antisense RNA molecules.

In addition, we designed a positive control plasmid (referred to as pLRH) that lacks the telomeric repeat sequences, but is otherwise identical to pHRT. In a circular form, the CMV promoter and RFP reporter of this plasmid are in the correct orientation to promote transcription of the RFP coding sequence.
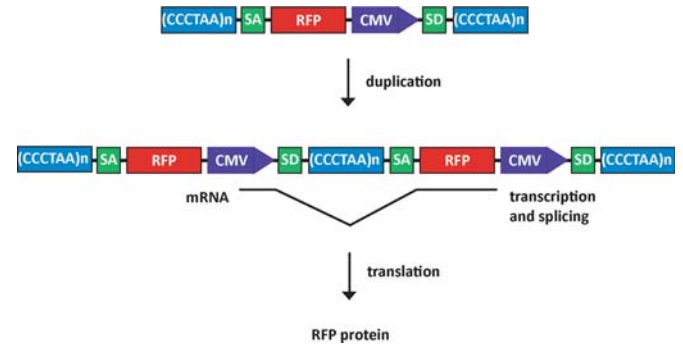

Figure 1. Description of the telomere-targeting construct. Graphical representation of the telomere-targeting construct (pHRT) in its linear form (not to scale). The RFP coding sequence is located upstream of a CMV promoter and will not be transcribed in this orientation. These sequences are flanked, respectively, by a splice acceptor and a splice donor site. At the 5′ and 3′ ends of the construct, we introduced 800 bp of telomeric repeats on each side, to promote integration at the telomere of host cells via homologous recombination. Telomeric sequences are shown as $(CCCTAA)_n$ to indicate integration of the construct at the C-rich telomeric DNA strand. If the construct is duplicated, the promoter and the reporter will be in the correct orientation for transcription of mRNA containing the coding sequence for RFP. The splice sites are also in the correct orientation to promote splicing of the telomeric sequences interposed between the two copies of the construct. This will ultimately result in the translation of RFP protein.

Detection of intra-telomeric sequence duplication in ALT-positive cells

The plasmids described earlier were transfected into the ALT-positive cell line IIICF/c (17). Cells transfected with the positive control pLRH plasmid and selected with hygromycin B started to show expression of RFP as early as 2 days after transfection and were still expressing the reporter when we ended our observation 66 days after transfection (Fig. 2, left panels).

The test plasmid pHRT was linearized as described in Figure 1 and transfected into IIICF/c. Colonies emerging from hygromycin selection were examined for RFP expression as soon as they were visible in the culture plates. We reasoned that the expression of the reporter at such an early stage of clonal growth could be a result of integration of the plasmid in tandem arrays rather than intra-telomeric duplication of the targeting construct and hence excluded such clones from further analysis (7 of 49 clones). Fourteen initially non-expressing clones were regularly monitored for the expression of RFP by fluorescence microscopy. In 13 of the clones, small and discrete clusters of fluorescent cells started to appear after ~14–25 population doublings (PDs; Fig. 2, right panels). For example, clone 3.1 started expressing RFP in a proportion of the cells after ~20 PDs and consistently expressed RFP in small and discrete clusters of cells throughout the experiment (which was continued for 152 days after transfection). Clone 1.4 started expressing RFP after ~21 PDs, with fewer clusters of fluorescent cells than clone 3.1. Clone 3.3 was monitored until it reached 35 PDs, but never showed expression of RFP.

As soon as sufficient cell numbers were obtained, we performed fluorescence *in situ* hybridization (FISH) analysis on metaphase spreads of all clones to map pHRT integrations (Fig. 3). Cells were hybridized with a pHRT-specific probe that consists of DNA sequences from the entire
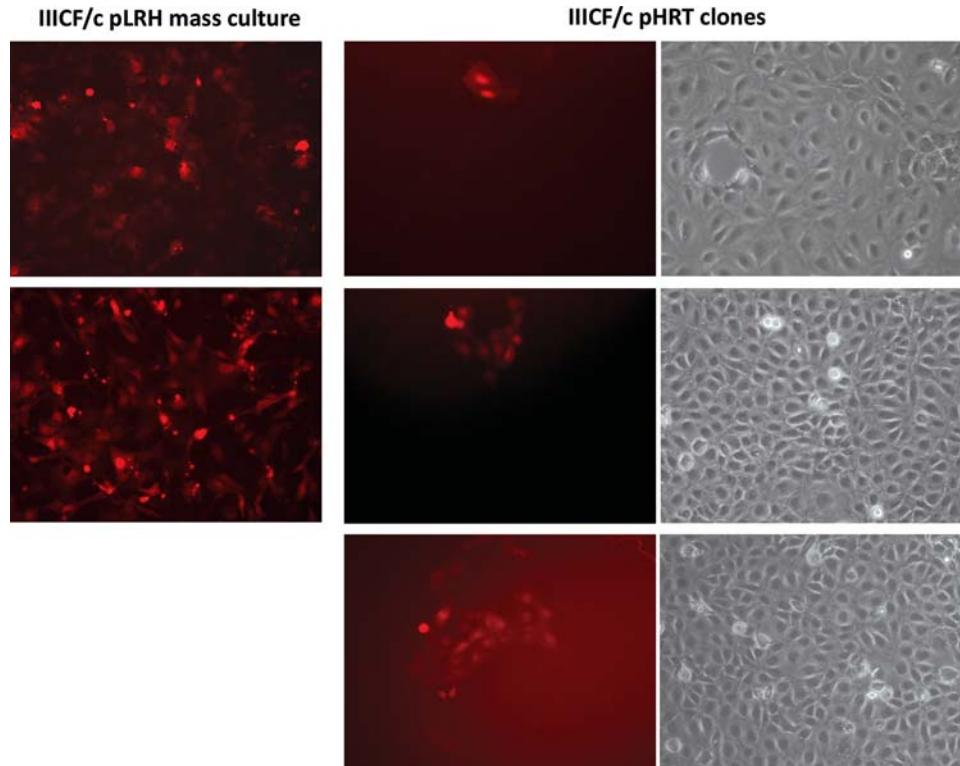
Figure 2. RFP expression in ALT-positive cells transfected with pHRT and pLRH. IIICF/c cells transfected with pLRH (positive control) showed diffuse expression of RFP as early as 2 days after transfection and kept expressing RFP throughout the observation time (left panels). In stable clones of IIICF/c transfected with pHRT, small and discrete clusters of RFP-positive cells were documented by fluorescence microscopy after a variable time of growth in culture, ranging from ∼14 to 25 PDs (right panels). In the right-hand panels, corresponding bright field and red fluorescence images are shown to visualize the clusters of RFP-positive cells among the non-fluorescent cells. Images were taken with a 20× objective.

pHRT plasmid, excluding the telomeric repeats to avoid hybridization at all chromosome ends. In clone 3.1, integration occurred at the telomere of the q arm of a sub-metacentric marker chromosome (arbitrarily referred to as 'marker chromosome A'). In ∼12% of clone 3.1 metaphases analyzed, we also observed integration at the telomere of the p-arm of an additional chromosome ('marker chromosome B'; data not shown). In order to test whether the telomere dynamics of clone 3.1 were typical of an ALT cell line and not altered by the integration of the pHRT construct, we carried out FISH analysis on metaphase spreads after this clone had completed 88 PDs, looking for spreading of the telomere tag as described previously (5). We found that the telomeric tag had been copied on to additional telomeres, consistent with the previous findings (data not shown).

In clone 1.4, the pHRT construct had integrated at the telomere of the q-arm of a large marker chromosome. Finally, in clone 3.3, which never showed expression of RFP, the pHRT plasmid was integrated at an interstitial site within the p-arm of another marker chromosome.

Confirmation of intra-telomeric sequence duplication

The clones in which pHRT integration had occurred at a telomere were able to produce RFP after a variable number of cell divisions, whereas the clone in which pHRT had integrated at an interstitial site was never able to express RFP. These data provide evidence that the pHRT construct has tandemly duplicated within the telomere in which it originally integrated (resulting in RFP expression) and that these tandem duplication events are restricted to telomere ends. However, due to the lack of sensitivity of FISH analysis, we were unable to confirm the presence of two or more copies of the pHRT construct in the clones that expressed RFP. Therefore, we developed a PCR–Southern blotting strategy to determine the number of copies of the pHRT construct on the same DNA strand, as depicted in Figure 4A.

As positive controls for the PCR reaction, we used a series of internal primer pairs that amplify within the construct and therefore result in amplification regardless of whether the construct is present in single or multiple copies. One of these amplicons partially overlaps with a DNA probe we designed for Southern analysis and was used as a positive control in the DNA blot. A second amplicon within the construct did not overlap with the DNA probe and was used as a control for non-specific hybridization of the DNA probe in the Southern blotting analysis (negative control).

As test reactions, we used primer pairs that are oriented away from each other within a single copy of the construct (R1–3 and F1–2, Figure 4A), so that amplification only occurs if tandem copies of the construct are present. The size of the amplicons is dependent on the length of the telomeric DNA tract intervening between copies of the construct. For most experiments, we used a common forward primer for all test reactions and reverse primers staggered
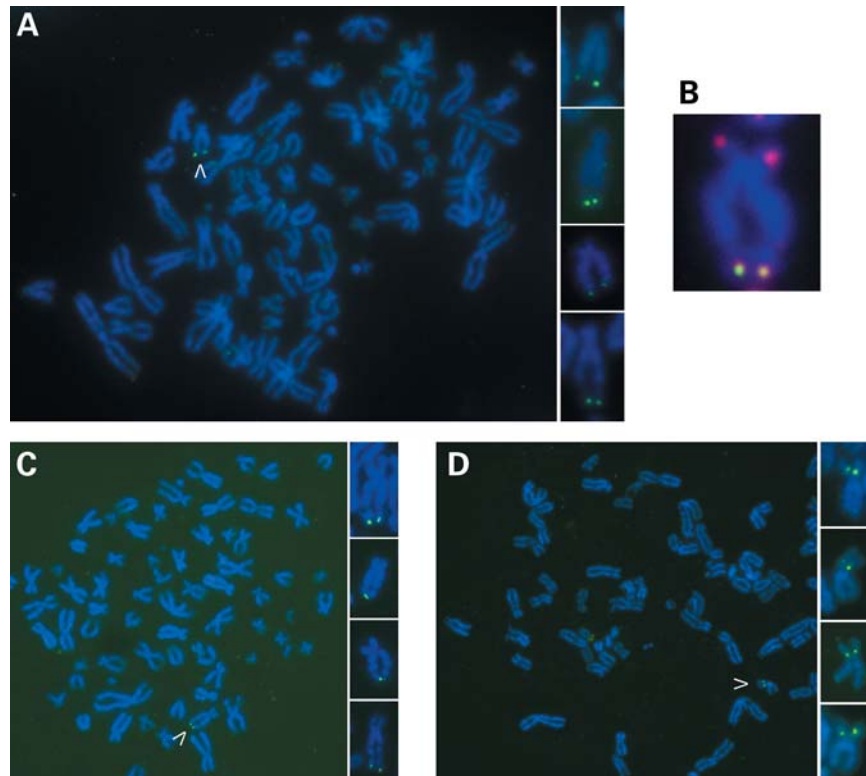
Figure 3. FISH analysis in IIICF/c pHRT clones. Metaphase spreads were obtained from IIICF/c pHRT clones and analyzed by FISH. Full metaphases and details of the chromosome tagged are shown. Cells were stained with DAPI to visualize the DNA (in blue) and probed with pHRT plasmid sequences devoid of the telomeric repeats (in green) to visualize the integration site. Early PD clone 3.1 revealed integration of the pHRT construct at the telomere of marker chromosome A (**A**). In (**B**), telomere-FISH (red) was combined with pHRT-FISH (green). The co-localization of the red and green signals and the presence of telomeric sequences proximal to the pHRT tag confirm telomeric integration of the pHRT tag. In clone 1.4, integration of pHRT was observed at the telomere of a marker chromosome (**C**). In clone 3.3, where fluorescence was never observed, integration of pHRT was interstitial within another marker chromosome (**D**).

along the length of the RFP coding sequence. The DNA probe used for Southern analysis overlaps with the amplification products of all test reactions.

Using this technique, we first analyzed genomic DNA extracted from clone 3.3, used as a negative control because the interstitial integration site was unable to express RFP. We also analyzed DNA obtained from clone 1.4 shortly after we were first able to visualize RFP-positive clusters of cells with fluorescence microscopy. Southern blots were obtained from the PCR products and hybridized either with a probe complementary to the pHRT plasmid, as indicated in Figure 4, or with the telomeric probe (TTAGGG)$_3$.

As shown in the PCR gels (Fig. 4), the positive and negative reactions generated amplicons of the correct size for both clones, and the pHRT probe only hybridized to the positive controls after Southern blotting. Test reactions F1−R1, F1−R2 and F1−R3 were designed to generate amplicons of at least 1100, 1300 and 1500 bp, respectively, in the presence of two copies of the plasmid and no amplification bands in the presence of a single copy of pHRT. For clone 3.3, no discrete amplicons were visible in the PCR gel or in the DNA blot (Fig. 4B). To exclude the possibility that there was no amplification due to a rearrangement of the construct during integration, we PCR-amplified across the construct and found that the priming site had not been affected and the fragment size was unchanged (data not shown). We therefore concluded

that the integrated pHRT construct was present (interstitially) as a single copy in clone 3.3.

In clone 1.4, the test reactions produced amplicons of 2600, 2800 and 3000 bp, respectively, and an additional, presumably spurious band of 1400 bp that appeared only in test reaction F1−R2 (Fig. 4C). With the exception of the 1400 bp band, all the amplicons hybridized both with the construct-specific probe and with the telomeric probe. This suggests that the PCR amplification reactions occurred across two copies of the pHRT plasmid, located on the same DNA strand and separated by ∼1500 bp of telomeric DNA. Considering that integration of pHRT in this clone was observed at the telomere of a single chromosome only, this result suggests that the plasmid DNA was duplicated by intra-telomeric processes. Based on the size of the amplicon, a duplication event might have occurred when the tract of telomeric DNA distal to the site of tag integration had shortened to <1500 bp. This event enabled the cells to produce RFP and occurred shortly before we first observed clusters of fluorescent cells within the clonal population (around 21 PDs).

Evidence for multiple independent intra-telomeric duplication events within a clonal cell population

To better understand the correlation between the ability to detect construct-derived amplicons and the expression of
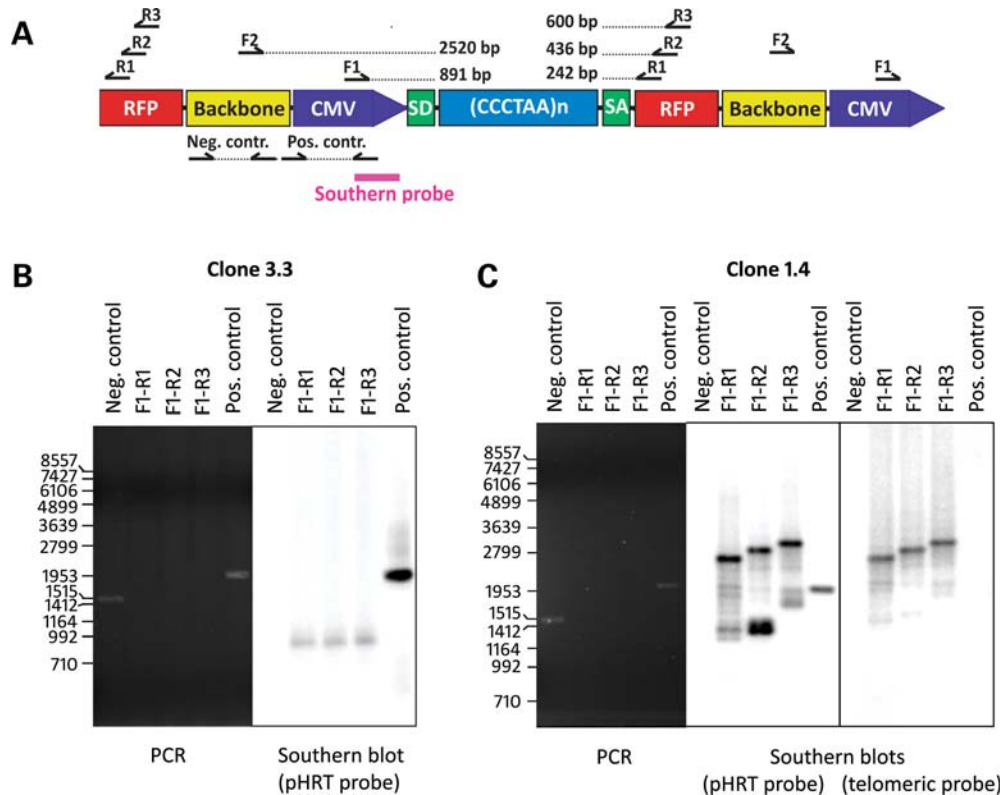
Figure 4. Molecular analysis of pHRT construct integration in IIICF/c pHRT clones 3.3 and 1.4. (**A**) PCR strategy for testing pHRT construct copy number in the IIICF/c pHRT clones. The forward and reverse PCR primers are oriented so that amplification only occurs if there is more than one copy of the construct. Size of PCR products will depend on the intervening telomere tract length (distances between each primer and the start of the telomeric repeat sequences are indicated). As positive and negative controls, we used primers amplifying a 1960 and a 1509 bp tract of DNA within the construct, respectively, the positive control overlapping with the Southern probe. (**B** and **C**) Clones 3.3 and 1.4 were analyzed by PCR–Southern blotting, with the probe depicted in (**A**). The test reaction primer pairs are indicated above each lane. The presence of bands of correct size in the control lanes of the PCR gels confirmed that these clones contained integrated pHRT DNA. In clone 3.3, the Southern blot probed for pHRT shows no discrete bands in the test lanes, indicating the presence of a single copy of the construct. In clone 1.4, Southern blotting analysis of the three test PCR reactions shows the presence of major amplicons of 2600, 2800 and 3000 bp. These amplicons contain pHRT (middle panel) as well as telomeric (right panel) sequences. This is consistent with amplification having occurred across two copies of the pHRT construct separated by ∼1500 bp of telomeric DNA.

RFP, we analyzed a population of RFP-positive cells obtained by fluorescence-activated cell sorting (FACS) of early passage cells from clone 3.1, soon after the RFP expression was first observed. First, we performed FISH analysis on sorted RFP-positive cells after a week of growth in culture and demonstrated that in 53% of the cells, the plasmid was integrated only in the telomere of marker chromosome A, i.e. there was reporter activity in cells in which the plasmid DNA had not been copied to any other telomere.

We then carried out the PCR–Southern blotting analyses described earlier. The test PCR generated three different amplicons in each reaction that hybridized both with the construct-specific probe and with the telomeric-specific probe (Fig. 5). The size of the amplicons suggested that at the time of observation, the plasmid tag was present as three different 'sets' of tandem copies, separated by tracts of telomeric DNA of ∼200, 700 and 1800 bp. We considered the following possible sources of the three amplicons of disparate length: (a) there could be four copies of the construct within the telomere; or (b) there could be three subpopulations of cells containing two copies of the construct. The first scenario could arise either from the integration of four copies of the construct into the same telomere at the time of transfection (this is very unlikely

because it would be expected to result in all cells within the population expressing RFP shortly after transfection), or from multiple duplication events. To distinguish between these possible scenarios, we analyzed early passage cells sorted for RFP expression at single-genome-equivalent amounts of DNA. We predicted that, if the first explanation was correct, we would be able to detect amplicons of different sizes within the same PCR amplification reaction. If the second hypothesis was correct, we expected to detect only one amplicon per PCR reaction and to detect amplicons of different sizes in different reactions.

IIICF/c cells are hypotetraploid (17), so assuming approximately 80 chromosomes per cell and therefore ∼10 pg of DNA per IIICF/c genome and 50% PCR efficiency, 20 pg of DNA was considered to be a single-cell equivalent for the purposes of this analysis. We serially diluted DNA from RFP-enriched clone 3.1 from 20 ng down to 20 pg and carried out PCR–Southern blotting analysis, as described earlier. The test reactions F2–R3, F1–R1, F1–R2 and F1–R3 are expected to produce amplicons of at least 3100, 1100, 1300 and 1500 bp length. As shown in Figure 5, the amplicons observed in previous experiments were amplified within the same PCR reaction when we used 20 ng, 2 ng and
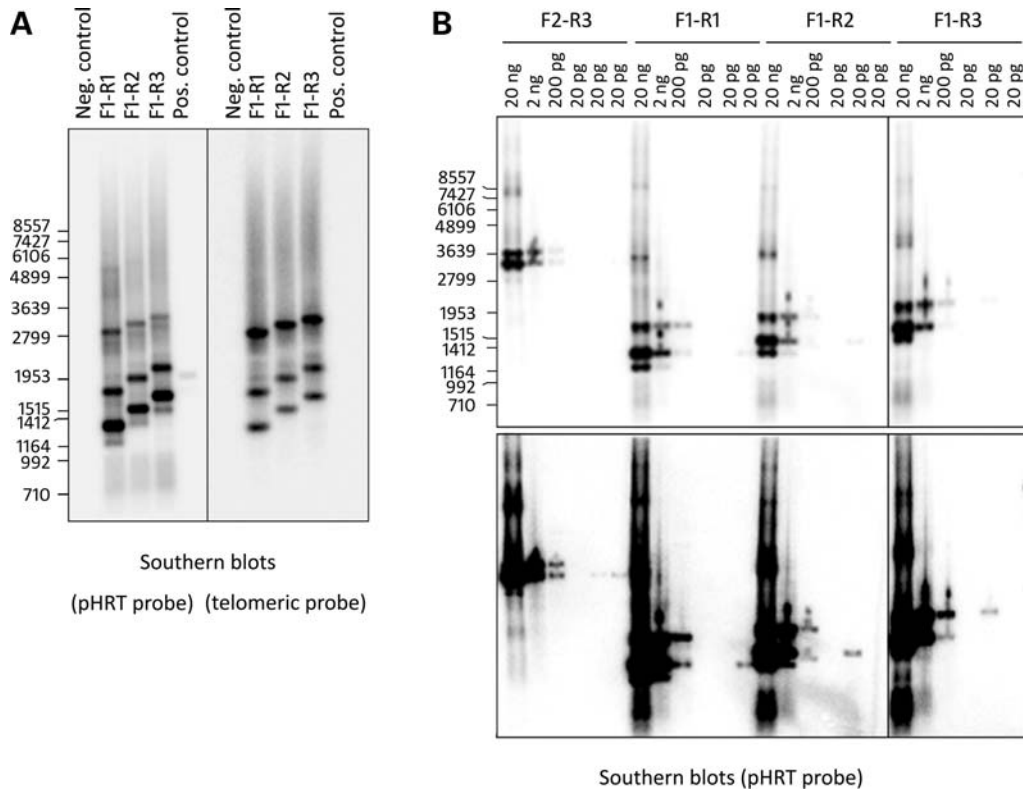
Figure 5. Molecular analysis of pHRT construct integration in IIICF/c pHRT clone 3.1. (**A**) Cells from IIICF/c pHRT clone 3.1 were enriched for HcRed expression by FACS. Southern blotting analysis of the three test PCR reactions shows the presence of three products for each amplification reaction. These amplicons contain sequences of the pHRT construct as well as telomeric sequences. The size of each amplicon is consistent with three separate two-copy-sets of the pHRT construct separated by 200, 700 and 1800 bp of telomeric DNA. (**B**) DNA from HcRed-positive IIICF/c pHRT clone 3.1 cells was serially diluted from 20 ng to 20 pg, the latter estimated as a single-genome-equivalent amount of DNA for these cells. The DNA was then PCR-amplified, as depicted in Figure 4, blotted and probed with pHRT sequences (F1–R3 amplification reactions were blotted on a separate membrane). The lower panel represents a longer exposure of the upper panel, to better visualize the amplicons obtained at 20 pg amounts of DNA (analyzed in triplicate for each set of primers). The two main amplicons, sized ~1300 and 1800 bp in reaction F1–R1, are amplified separately when the DNA is diluted to single-genome equivalents, suggesting that they are located within separate telomeres.

200 pg of DNA, corresponding to 1000, 100 and 10 genome equivalents. Scaling the amounts of DNA down to single genome equivalents (20 pg of DNA), we were able to amplify only the two major amplicons, sized ~1300 and 1800 bp in test reaction F1–R1 and the corresponding larger amplicons in the other test reactions. In all five reactions in which an amplicon was generated, we observed either the larger or the smaller product, but never observed both in the same reaction. We therefore concluded that the amplicons are located on separate DNA strands and are the result of independent duplication events occurring in different cells. One cell population has constructs separated by ~200 bp of telomeric DNA (~30 repeats), whereas the other has constructs separated by ~700 bp of telomeric DNA (~120 repeats). This result also excludes the possibility that multiple copies of the pHRT construct integrated at the time of transfection.

Absence of intra-telomeric duplication events in clones obtained from a telomerase-positive cell line

To assess whether intra-telomeric duplication events are exclusive to the ALT mechanism or a common feature of immortal cell lines, we transfected the telomerase-positive

cell line HT1080 with the telomere-targeting construct pHRT. We obtained five clones and monitored them by fluorescence microscopy for 38–45 PDs. None of the five clones expressed RFP during the observation period. FISH analysis confirmed that the pHRT construct had integrated at a telomere (clone 22, Fig. 6A). The PCR–Southern blotting analysis carried out as described previously on DNA pooled from clones 22 and 23 did not produce any amplicons in the test reactions F1–R1, F1–R2 and F1–R3, but produced an amplicon of the correct size for the positive control reaction (Fig. 6B). This confirmed that the pHRT construct was still present in single copy in cells from HT1080 clones 22 and 23.

DISCUSSION

We have demonstrated that intra-telomeric duplication of DNA sequences occurs in ALT-positive cells. This was shown initially by a reporter assay designed to detect intra-telomeric duplication. Clones that expressed the reporter shortly after integration were excluded from the analysis, because in these clones the expression could have resulted from rearrangement of the construct during integration or from integration of the construct in tandem arrays. As expected
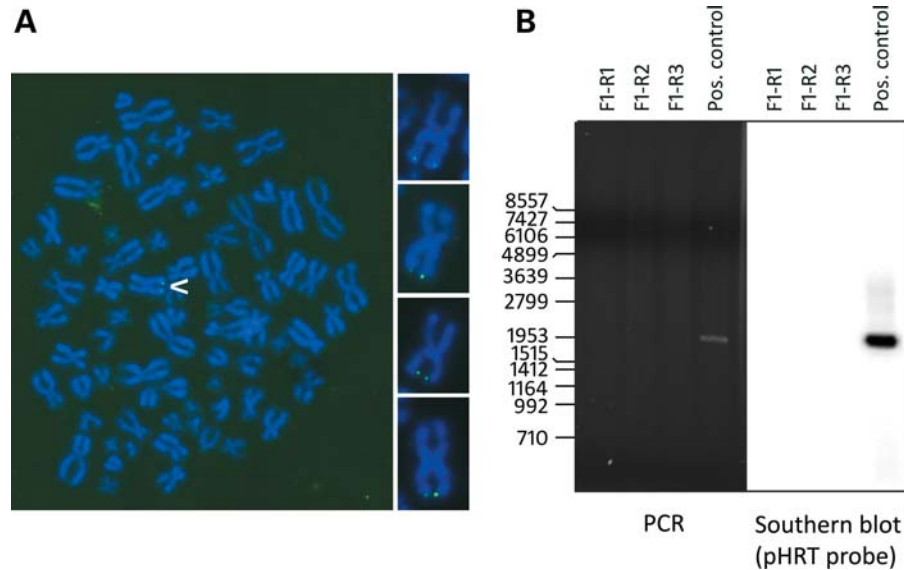
Figure 6. (**A**) Analysis of HT1080 clone 22 and 23 cells by FISH and PCR. FISH analysis performed on metaphase spreads obtained from HT1080 clone 22 cells (telomerase-positive) indicated that integration had occurred at the telomere of the q arm of a large chromosome. (**B**) PCR and Southern blotting were carried out on DNA pooled from clones 22 and 23, as described earlier. The internal primer pair used as a positive control produces an amplicon of correct size that contains construct-specific sequences, confirming its integration in the genome.

based on our previous study that showed inter-telomeric copying in ALT cells (5), the construct was copied on to other telomeres after a variable time period. After this had happened, a duplication event within a telomere could potentially be explained by copying from one telomere to another (i.e. copying from the new telomeric site back to the original telomeric site where the construct remains or vice versa). We therefore restricted our analyses to the time interval before inter-telomeric copying events were detected or became widespread. Fourteen clonal cultures that were initially negative for reporter activity were chosen for observation, and islands of reporter-positive cells appeared in 13 of these clones after 14–25 PDs. It was subsequently shown that the remaining clone did not have a telomeric copy of the reporter construct, but instead had integrated it into an interstitial chromosomal site. In contrast, none of the five clones of a telomerase-positive cell line with telomeric integration of the construct acquired reporter activity. These data are consistent with the integration of the construct as a single copy, followed by generation of one or more additional adjacent copies in a minority of cells within an ALT-positive, but not a telomerase-positive, cell population after a variable period of time.

One of the 13 clones was sorted for reporter expression, and the FISH analysis revealed that in >50% of the expressing cells, the reporter construct was present exclusively at the originally tagged telomere. This indicates that the most likely source for generating an additional copy of the construct in these cells is the tagged telomere itself through an intra-telomeric DNA amplification mechanism. This clone and one other of the 13 clones were chosen for molecular analyses, together with the clone with interstitial integration as the negative control. PCR amplification and Southern blotting confirmed that these two clones had tandem copies of the reporter separated by telomeric DNA. As expected from the absence of reporter activity, tandem copies were not detected

in telomerase-positive cells. PCR amplification of single-cell equivalent DNA from a reporter-positive ALT clone demonstrated that there had been different duplication events within separate cells in the population.

We consider the three most likely explanations for the observed intra-telomeric duplication to be unequal t-SCEs, copying of telomeric sequences between sister chromatids and/or loop-mediated copying. t-SCE rates are increased in ALT cells, but there has not previously been any evidence that they might be unequal, with the net gain of telomeric material on one sister chromatid and net loss on the other. It has been suggested, however, that this could contribute substantially to telomere length maintenance in ALT cells (10), and it was subsequently suggested (18) and confirmed by mathematical modeling (19) that telomere maintenance could occur by this means if there is also a mechanism to segregate the lengthened telomeres asymmetrically into one daughter cell. An unequal exchange of telomeric sequences in two sister chromatids could result in one chromatid gaining a copy of the construct at the expense of the other. The daughter cell that acquires the latter chromatid would not have the selection marker (HPT) and would be lost from the cell population that was exposed continuously to hygromycin. In four ALT cell lines, the frequency of t-SCEs ranged from 0.28 to 2.8 per metaphase (8). If the frequency is assumed to be ~1.6 per cell division and there are approximately 160 chromosome ends per IIICF/c cell, then the frequency of a t-SCE involving the tagged telomere should be once per 100 cell divisions. The observation that the reporter activity did not appear for 14–25 PDs (14 PDs is equivalent to >16 000 cell divisions) indicates that if the reporter activity is in fact due to unequal t-SCEs, then the frequency of these events is much lower than the observed frequency of t-SCEs. This could indicate that the proportion of t-SCEs that are unequal is low and/or that the proportion of unequal

t-SCEs that result in two adjacent copies of the construct capable of generating reporter activity is low.

Another possible explanation for duplication events at the telomere is copying of DNA between sister chromatids. If the telomere of one sister chromatid becomes critically short due, for example, to a rapid deletion event, the other sister chromatid could be used as a source of telomeric sequences for elongation. If strand invasion occurs on the centromeric side of the telomeric tag of the sister chromatid, new DNA synthesis will result in duplication of the tag.

It is also possible that the duplication events are due to loop-mediated copying: once the 3′ single-stranded overhang folds back and invades the duplex DNA, it could simply elongate by leading strand DNA synthesis, using the complementary strand as a template; the C-rich strand could subsequently be elongated by lagging strand synthesis. The size of t-loops in ALT cells ranges from 0.5 to 70.2 kb (13). On the basis of size alone, there is no reason to exclude the possibility that a loop could form that includes the entire integrated reporter construct. The ability of telomeres containing a relatively large tract of non-telomeric sequence to form a loop has not actually been tested, but critical determinants of t-loop formation include the presence of TTAGGG double-stranded (ds) and single-stranded (ss) sequences at the ds/ss junction of the telomere terminus (20). The minimum length of telomeric ss overhang is ∼50 nt (21), and the length of the D-loop resulting from the insertion of the ss overhang into the duplex telomeric DNA may be 75–200 nt (7). Therefore, if the tagged telomere underwent shortening so that the tag was now close to the terminus and a loop formed that mostly consisted of construct DNA, it may be expected that the minimum amount of $(TTAGGG)_n$ DNA in the loop would be in the range of 75–200 bp. If this loop then primed DNA synthesis, resulting in duplication of the construct DNA within the telomere, then the minimum length of the tract of $(TTAGGG)_n$ DNA between the constructs would be 75–200 bp. Interestingly, the smallest distance between constructs detected by PCR–Southern blotting analysis was ∼200 bp. Although loop-mediated telomere extension remains an intriguing possibility, the analyses cannot discriminate between this and sister chromatid-mediated duplication events.

We excluded from the analysis any clones that expressed the reporter shortly after transduction on the basis that, in these clones, the construct may have undergone rearrangement during the integration event or multiple copies of the construct may have integrated as a tandem array. Nevertheless, we considered the possibility that the clones that were initially negative for reporter expression contained a tandem array from which expression was initially repressed but later became derepressed in a minority of cells within the population due to a stochastic event. Transcriptional repression of telomerically located sequences is well documented in yeast species (22) and is referred to as the telomere position effect (TPE) (23). Studies of TPE in human cells have analyzed the transcription of reporter genes inserted by flanking them with telomeric repeats only on one side, so that their stable integration causes breakage of a chromosome arm and 'seeding' of a new telomere, resulting in the reporter being located immediately proximal to telomere sequences and distal to sequences that are not normally in a subtelomeric location (24–26). This contrasts with our experimental approach, in which the reporter was flanked with telomeric repeats on both sides for targeting the construct intra-telomerically. It is important, nevertheless, to note that the results of the TPE studies indicate that reporter sequences can be repressed when integrated in close proximity to a telomere. However, the silencing effect started only after the construct had become stably integrated in the host cell and resulted in a gradual decrease in reporter expression until low but stable expression levels were reached (26). The levels of reporter expression were totally independent of telomere length at the time of integration in both telomerase-positive (26) and ALT-positive (24) cells. Growth in selective medium has been shown to maintain stable levels of transgene expression in mouse embryonic stem cells (27). On the basis of these data, if the reporter construct had integrated in multiple copies in our clones, we could expect to detect the expression of the reporter early after transduction followed perhaps by a gradual decrease, but not to zero because of continued growth in selective medium. Similarly, if reporter expression was the result of transcription of telomeric sequences on the C-strand (referred to as ARRET) (28), it would have appeared soon after transfection and in most cells of the clonal populations. In contrast, we observed the absence of expression early and expression in isolated clusters of cells after 14–25 PDs.

Another possibility we considered is that circular ECTR DNA containing the construct sequences could be used as a template for multiple rounds of elongation via the 'rolling circle' mechanism. Telomerase-deficient strains of *Kluyveromyces lactis* can elongate their telomeres by using transforming DNA circles as copy templates (29). Untransformed clones can also elongate their telomeres by 'rolling circle', and the source of the circular DNA in these cells is thought to be one of the telomeres (29). In mammalian cells, the occurrence of ECTR-mediated telomere elongation has not yet been demonstrated. Large circles of ECTR DNA are abundant in ALT cells (13,14), and their size is proportional to the telomere length in these cells (14). It has been speculated that ECTR circles are generated by resolution of a t-loop structure (14), and a similar mechanism has been proposed to explain telomere rapid deletion events (30). In the clones we analyzed, amplification of the tag via rolling circle replication would have required the formation of a circular molecule encompassing the entire plasmid sequence (∼7 kb excluding the $(TTAGGG)_n$ arms). Excision of this large t-loop structure at the junction point would generate a shortened telomere without plasmid sequences and a large extra-chromosomal circle containing the plasmid sequences. The circle could then be used to elongate any telomere, enabling the cells to produce RFP if more than one copy was made. However, when we compared an unsorted population of cells from clone 3.1 with a population sorted for RFP expression, we found that the majority of the cells in both populations retained the tag on marker chromosome A only. If rolling circle-mediated amplification of the tag had occurred, the only possible explanation is that the circle was copied back as an array on the same telomere from which it originated, but was not used as a template for elongation by other telomeres. Although, in yeast, short telomeres are
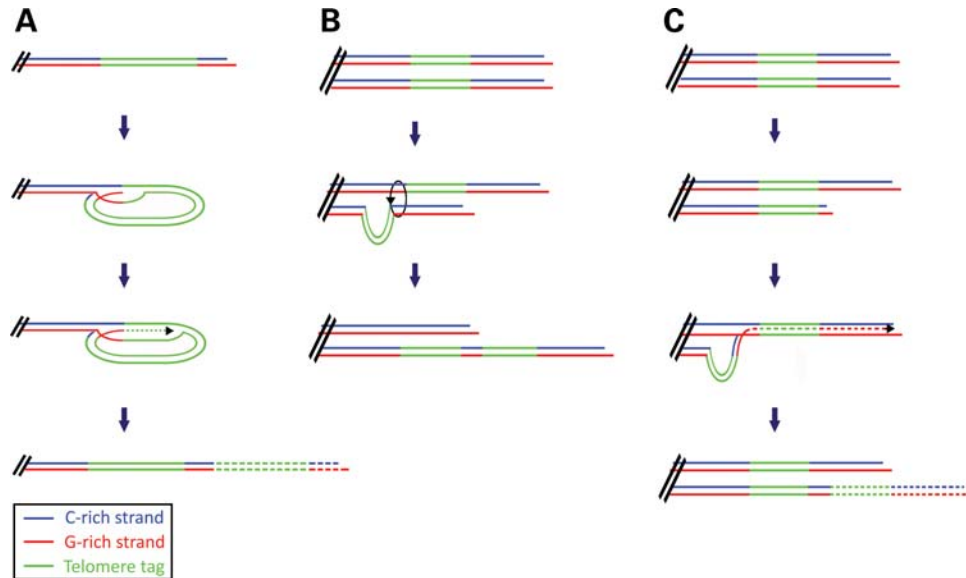
Figure 7. Potential mechanism generating intra-telomeric duplication events. Graphical representation of three proposed mechanisms for duplication of a telomeric tag. (**A**) Loop-mediated DNA copying. The tagged telomere folds back and forms a loop structure consisting mostly of construct DNA. If the loop configuration primes new DNA synthesis, this results in duplication of the telomere tag. (**B**) Unequal t-SCE. Telomeric sequences distal to the construct DNA of one sister chromatid anneal to the complementary strand proximal to the construct DNA in the other sister chromatid. If this is followed by an unequal exchange, one sister chromatid will gain an additional copy of the construct, whereas the other will lose the construct sequences (and the resulting daughter cell will be lost from the population. (**C**) Sister chromatid copying. Only one sister chromatid undergoes rapid shortening of the telomeric DNA distal to the construct and becomes highly recombinogenic, whereas the other sister chromatid maintains a relatively long telomere. The short 'distal' telomere anneals to the 'proximal' telomeric DNA of its sister chromatid and this is followed by new DNA synthesis. This results in one sister chromatid gaining an additional copy of construct, whereas the other sister chromatid maintains a single copy. Dotted lines: newly synthesized DNA.

highly recombinogenic (31), there is no evidence that the same telomere is involved in generating and then copying the extra-chromosomal circle. Moreover, rapid elongation has been observed in long as well as in short telomeres in mammalian ALT cells, suggesting that human ALT telomeres may be recombinogenic regardless of their length (3). For these reasons, we do not regard a rolling-circle mechanism as the most likely source of the reporter construct amplification.

In conclusion, we found that ALT cells are able to duplicate a tag inserted at their telomeres without any apparent intervention of other telomeres. This intra-telomeric duplication may have occurred by one of several mechanisms (Fig. 7), including t-loop-mediated DNA synthesis, unequal t-SCEs and copying of telomeric sequences between sister chromatids. This study demonstrates that, in addition to the inter-telomeric copy templating documented previously, the telomere dynamic characteristics of ALT cells include intra-telomeric elongation events.

MATERIALS AND METHODS

Plasmids

The positive control plasmid pLRH was obtained as follows. We excised the zeomycin resistance cassette from the pSHM06-TPI vector (kindly provided by Prof. Melissa Moore, University of Massachusetts Medical School, Worcester, MA, USA) and substituted it with an HPT cassette obtained from pTK-Hyg (Clontech Laboratories, Inc.) using $NsiI + BsmI$ (pSHM06-TPI), $NruI + HindIII$ (pTK-Hyg), End-It^{TM} DNA End-Repair Kit (Epicenter Biotechnologies) to create blunt

ends, followed by religation. We then substituted the *Renilla Luciferase* coding sequence with HcRed, obtained from pHcRed1-N1/1 (Clontech Laboratories, Inc.) using $XbaI + BstBI$ (vector), $XbaI + AccI$ (pHcRed) and religation. The pLRH plasmid was transfected in a circular form. To obtain the pHRT plasmid, we first eliminated a $KpnI$ site from pLRH using $SmaI + MscI$ digestion, gel purification of the vector and religation. We then opened the vector within the TPI intron 6 sequence and inserted 1.6 kb of telomeric repeats excised from pSXneo-1.6T$_2$AG$_3$ (kindly provided by Prof. T. de Lange, Rockefeller University, New York, NY, USA). This insert contains a multiple cloning site in the middle, with two $KpnI$ restriction sites. For transfection, we linearized the pHRT plasmid using $KpnI$ and gel-purified the corresponding fragment to promote telomeric integration.

Cell culture

IIICF/c cells were obtained as described previously (17). For transfection with either pLRH (positive control) or pHRT plasmid DNA, IIICF/c cells were plated at a density of 1×10^6 cells per 10 cm culture plate. Twenty-four hours later, cells were incubated with 1.5 µg of pLRH or pHRT plasmid DNA and 30 µl of Fugene 6 Transfection Reagent (Roche) in 1 ml OPTI-MEM serum-free medium (Gibco) at 37°C. After 2 h, 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum was added to the transfection mixture, and the cells were incubated at 37°C. Twenty-four hours later, cell were trypsinized and plated at a density of 5×10^3 cells per 10 cm culture plate.

Selection with 150 µg/ml hygromycin B was started 24 h later, and clones were isolated after 10–14 days, when the untransfected cells had died. Clones were monitored for RFP expression by fluorescence microscopy (Leica DMIRB microscope with appropriate filter sets) before isolation and one to three times per week after isolation.

Sorting

HcRed-positive cells were initially identified using a FACS-Canto (BD Biosciences) flow cytometer. Cells were excited with the He–Ne laser 633 nm line and the emission was captured through a filter/bandpass (nm) 660/20 (Living Colors User Manual Volume II. Protocol No. PT3404-1, Version No. PR37085, Clontech Laboratories, Inc).

Sorting HcRed-positive cells was performed on a FACS-Vantage SE (BD Biosciences) with a 570–600 nm tunable laser. When passed through a rhodamine dye, the primary green laser (532 nm) is shifted to ~590 nm, which is near the excitation maximum of HcRed (588 nm). Cells at a density of 2.8–6 million/ml were pre-filtered through 35 mm strainer mesh. For sorting, a nozzle size of 130 mm and a low sort rate were used. Stringent sort parameters were used to eliminate aggregates and ensure optimal purity of the HcRed-positive sorted population.

FISH

FISH analysis was performed essentially as described previously (5). Metaphase chromosome images were obtained using an Olympus BX51 microscope with a $100\times$ oil objective (NA = 1.35) and appropriate filter sets and merged using SPOT 4.4 software. To increase the number of images collected, we used an AxioImager M1 automated microscope (Carl Zeiss) with a $63\times$ oil objective (NA = 1.4), appropriate filter sets and Metafer (Metasystems, Germany) metaphase finder software. For chromosome identification, we used the diamidino-phenyl-indole-dihydrochloride (DAPI) banding pattern. For illustrative purposes, the intensity of the counterstains (DAPI and propidium iodide) was modified using Adobe Photoshop 6.0, but no processing was applied to the green color channel [corresponding to the fluorescein isothiocyanate (FITC)-detected plasmid tag]. For each clone, between 67 and 209 metaphases were analyzed. For dual color FISH experiments, we detected the integrated pHRT plasmid first with two layers of avidin-FITC (Vector Laboratories), followed by cross-linking in 4% formaldehyde and then denaturation and hybridization with Cy3-conjugated telomere-specific PNA probe (Applied Biosystems).

PCR–Southern blotting

We used 200–400 ng of genomic DNA for each standard PCR reaction, depending on sample availability. For single-molecule PCR, we used serial dilutions of genomic DNA from 20 ng to 20 pg, the latter reaction in triplicate. To ensure accurate dilution of the samples, we digested the genomic DNA with *Nsi*I, a restriction enzyme that leaves both the telomeric repeats and the pHRT sequences intact. To avoid loss of material during sample preparation, we used DNA from the SL2 cell line (derived from *Drosophila melanogaster* and kindly donated by Prof. Sharad Kumar, Institute of Medical and Veterinary Science, Adelaide, Australia) as a carrier. As a control for this experiment, we included PCR amplification reactions of genomic DNA from SL2 cells and from untransfected IIICF/c cells (20 ng each). None of the test or control reactions produced an amplicon (data not shown). After PCR amplification, products were resolved on agarose, stained with ethidium bromide and photographed under UV light. Gels were Southern-blotted, hybridized with a 366 bp DNA probe complementary to the pHRT plasmid (as described in Fig. 4) and radiolabeled with α^{32}P-dCTP by random priming. Parallel DNA blots were obtained from each PCR amplification and hybridized with the telomeric probe $(TTAGGG)_3$, end-labeled with γ^{32}P-ATP. Membranes were washed and exposed to Phosphor screens (Molecular Dynamics).

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