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Detection of circular telomeric DNA without 2-D gel electrophoresis

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Abstract

The end of linear chromosomes form a lasso-like structure called the t-loop. Such t-loops resemble a DNA recombination intermediate, where the single-stranded 3' overhang is arrested in a stretch of duplex DNA. Presumably such a t-loop can also be deleted via a recombination process. This would result in the occurrence of circular extra-chromosomal telomeric DNA (t-circles), which are known to be abundantly present in immortal cells engaging the recombination-based alternative lengthening of telomeres pathway (ALT pathway). Little is known about the basic mechanism of telomeric recombination in these cells and what ultimately causes the generation of such t-circles. Current standard procedures for detecting these molecules involve 2-D gel electrophoresis or electron microscopy. However, both methods are labor-intense and sophisticated to perform.

We would like to present a simpler, faster and equally sensitive method for detecting t-circles. Our approach is a telomere restriction fragment assay that involves the enzymatic preservation of circular DNA with Klenow enzyme followed by Bal31 degradation of the remaining linear DNA molecules. We show that with this approach t-circles can be detected in ALT cell lines, whereas no t-circles are present in telomerase-positive cell lines. We consider our approach being a valid method that can be used in experiments, in which t-circle generation is the experimental read out.

Introduction

Cellular immortality is dependent on a mechanism of telomere length control by which cells are able to compensate for the loss of telomeric sequences that is associated with each cell division (Murnane J. P., 2006; Watson J. D., 1972). To preserve genomic integrity stable telomeres are required, which is achieved when a higher-order structure called the t-loop is formed (de Lange T., 2002; Griffith J. D. et al., 1999; Munoz-Jordan J. L. et al., 2001). This loop formed by the chromosomal end involves the canonical TTAGGGn repeats with their 3' single-stranded overhang as well as telomere-binding proteins (Karlseder J., 2003). For loop formation the single-stranded overhang is sequestered in a short sequence of duplex telomeric DNA (d-loop). The t-loop model is a model that explains telomeric end-capping by which the linear chromosomal end is not recognized as a DNA double-strand break, hence preventing growth arrest, DNA recombination and genetic instability (de Lange T., 2005; Takai H. et al., 2003).

The t-loop structure resembles an intermediate in homologous recombination. The single-stranded overhang which is tucked in a short stretch of displaced double-stranded telomeric DNA (D-loop) resembles a Holliday junction. Proteins involved in homologous DNA recombination, e.g. RAD51D, are found to be part of the telomere protein complex (Tarsounas M. et al., 2004). Resolution of t-loops via overexpression of a mutant TRF-2

protein results in circular telomeric DNA molecules which suggests that t-loops can be a substrate for telomeric deletions via a recombination mechanism (de Lange T., 2004; Wang R. C. et al., 2004). Finally, extrachromosomal circular telomeric DNA is frequently found in cells engaging the recombination-based alternative lengthening of telomeres (ALT) pathway, suggesting that t-circle formation is part of the mechanism of ALT cells (Cesare A. J. et al., 2004; Henson J. D. et al., 2002; Lustig A. J., 2003).

Understanding telomeric recombination and the basic mechanism central to the ALT pathway is important for several reasons. There is still a lack of understanding about what leads to ALT pathway activation. As a consequence, ALT can not be diagnosed in a precise manner. Currently, ALT is defined when cells have long and heterogeneous telomeres, when any detectable telomerase activity is missing and ALT-associated PML bodies are present (Bryan T. M. et al., 1995). Although the vast majority of human tumors depend on telomerase activity as the preferred pathway of telomere length control, ALT can be found in a substantial number of sarcomas (Johnson J. E. et al., 2007). Since ALT is diagnosed only when the telomerase pathway is firmly ruled out, one cannot exclude the possibility that tumors exist, where certain areas are ALT-positive and other areas contain tumor cells that have engaged the telomerase pathway. This is important when anti telomerase therapy is considered in future cancer treatment strategies. Selection of per se treatment-resistant cells and engagement of telomeric recombination are issues that need to be considered (Bechter O. E. et al., 2004).

It is important that we have a simple method for detecting t-circles as a feature of the ALT pathway. This can make telomere maintenance diagnosis easier, especially when a larger volume of clinical samples is analyzed. It can also facilitate experiments where t-circle generation is the experimental read-out. Current methods for detecting such circles are 2-D gel electrophoresis or electron microscopy. However, both procedures are labor-intensive and sophisticated to perform (Cesare A. J. et al., 2004; Wang R. C. et al., 2004). We therefore sought to establish an easier and faster protocol for t-circle detection. Our approach is based on enzymatic preservation of circular structures followed by degradation of linear DNA molecules prior to 1-D gel electrophoresis. With this protocol we show that extra-chromosomal circular telomeric DNA can be detected in ALT cell lines, but is lacking in telomerase-positive cell lines or in ALT cells where the ALT pathway is blocked by ectopic expression of telomerase.

Material and Methods

Cell lines

All cells were grown at 37°C in Dulbecco's modified Eagle's medium (PAA Laboratories, Etobicoke, Ontario, Canada) including 10% fetal calf serum (Sigma Aldrich, St. Louis, Missouri, USA) plus standard antibiotics. The human breast cancer cell line MCF-7, the cervical cancer cell line Hela and the colorectal cancer cell lines SW480 and Lovo are telomerase positive and were obtained from the ATCC cell repository. VA-13, SW26 and SW39 cells are human fibroblasts that have been immortalized by simian virus 40 large T antigen expression and were a kind gift of Drs J. Shay and W. Wright (Department of Cell Biology, UTSouthwestern Medical Center, Dallas TX). VA-13 and SW26 cells use the ALT pathway, while SW39 cells use telomerase for telomere maintenance. The Susmi and Saos cell lines are ALT cell lines obtained from the ATCC cell repository. VA-13 hTERT#1 and VA-13 hTERT#7 are VA-13 ALT cell clones that have hTERT overexpressed and are characterized as ALT cell lines in which the ALT pathway is blocked by telomerase expression (Ford L. P. et al., 2001).

TRF analysis

To obtain nucleic acid, DNA extraction was done following the protocol of the Purgene DNA extraction kit (Gentra Systems, Minneapolis, Minnesota, USA). The amount of DNA obtained was measured with a photometer (Eppendorf, Hamburg, Germany). For telomere length analysis 2 µg of genomic DNA was digested overnight with the enzymes RsaI and HinfI (1x NEB 2, total volume 25µl; New England Biolabs, Beverly, MA, USA) at 37°C. Digested DNA was run on a 0.8% agarose gel in 1x TAE buffer at 1.4V/cm. After denaturing (0.5 M NaOH, 1.5 M NaCl) for 20 minutes the gel was rinsed in H₂O for 10 minutes, dried at 56° C for three hours and finally neutralized (1.5 M NaCl, 0.5 M Tris). After neutralization, hybridization with a P³²-labeled (TTAGGG)₄ oligonucleotide (Metabion, Martinsried, Germany) was performed at 42°C for 16 hours. After washing the gel once in 2 × SSC (1 × = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) for 15 minutes at room temperature followed by two washes with 0.1 × SSC/0.1% SDS for 10 minutes at room temperature the gel was exposed to a phosphor screen and analyzed on a phosphor imager (Cyclone Storage Phosphor System, Perkin Elmer, Waltham, MA, USA). For gels that were hybridized with an oligo against a microsatellite region (GCTGGAAGTTCTGGCCAG; Metabion, Martinsried, Germany) the same hybridization and washing conditions were applied.

Nuclease treatment and T-circle protocol

Ten µg of VA-13 ALT cell DNA was digested overnight at 37°C with RsaI and HinfI (1x NEB 2, total volume 25µl; New England Biolabs, Beverly, MA, USA) after which the reaction mixture was heat-inactivated at 80°C for 20min. For Klenow fill-in the samples were subjected to a reaction containing 33µM dNTPs, 2U Klenow enzyme large fragment (New England Biolabs, Beverly, MA, USA) in 1x restriction enzyme buffer in a total volume of 30µl at 25°C for 15min, followed by heat inactivation at 75°C for 20min. DNA ligation was performed in 1x T4 DNA ligation buffer with 5U of T4 DNA ligase (Roche Biodiagnostics, Vienna, Austria) at 20°C for 30min followed by heat inactivation at 65°C for 10min. For Bal31 degradation of the remaining linear DNA molecules 0.5U Bal31/µg DNA was used at 30°C in 1x buffer in a total volume of 40µl (New England Biolabs, Beverly, MA, USA). For T7 endonuclease treatment 2U enzyme/µg of DNA was used for 6h at 37°C in 1x NEB 2 (New England Biolabs, Beverly, MA, USA). Subsequently the samples were processed following a regular TRF protocol as described above.

Telomere separation protocol

For separation of telomeres from the bulk genomic DNA we followed the protocol published by Wright et al (Wright W. E. et al., 1997). For each assay, 40µg of genomic DNA was predigested with RsaI and HinfI and subsequently subjected to an annealing reaction to a biotin-labeled C-rich oligo (AATCCC)₆ (Metabion, Martinsried, Germany) at decreasing temperatures of 80°C, 65°C, 55°C, 45°C, 35°C for 30min each in 1xSSC/0.1% Triton X-100. After the annealing reaction streptavidin-coated magnetic beads (20µl of a 10mg/ml stock solution) (New England Biolabs, Beverly, MA, USA) that were pre-washed (1xSSC/1% Triton X-100, coated with 5x Denhardt's for 30min at RT) and resuspended in 1x SSC were added to the reaction mixture. The samples were incubated overnight at 4°C in a rotator to keep the beads in suspension. To recover telomeres, beads were gently washed twice in ice cold 1x SSC/1% Triton X-100. For collection of the beads during each washing step and for telomere recovery an ice-cold magnet was used. To elute the retrieved telomeres, the beads were brought into suspension with 50µl of 1xTE buffer and incubated at 65°C for 10min. For detection of telomeric DNA a regular TRF protocol was applied at the end of the procedure.

2-D gel electrophoresis

For 2D gel electrophoresis we followed the protocol described by Wu et al. (Wu L. et al., 2006). With this procedure DNA molecules are separated by size (1st dimension) as well as structure (2nd dimension). Briefly, 20µg of genomic DNA from VA13 and SW39 cells was digested with RsaI/HinfI (1x NEB 2, total volume 25µl; New England Biolabs, Beverly, MA, USA). For indicated samples an additional t-circle protocol was conducted as described above. Separation in the first dimension was done in a 0.4% agarose gel in 1x TBE buffer without ethidium bromide at 1V/cm for 18h. After staining with ethidium bromide, lanes were cut out and cast in a 1.1% agarose gel at a 90° orientation from the first gel and resolved at 5V/cm at 4°C in 1x TBE buffer supplemented with 0.3µg/ml ethidium bromide with constant buffer circulation. In gel hybridization was identical to the regular TRF procedure. For comigration analysis 1µg of the ampicillin-containing circular plasmid pEGFP-N3 (Clontech, Palo Alto, CA, USA) was admixed to the genomic DNA and subjected to gel electrophoresis. To detect the circular plasmid, gels were hybridized with an ampicillin-specific probe.

Results

Klenow treatment preserves telomeric DNA from Bal31 degradation

In order to establish the reaction conditions necessary for eliminating all linear telomeric sequences, we subjected 10µg of HindIII-digested (New England Biolabs, Beverly, MA, USA) genomic DNA from telomerase-positive MCF7 as well as VA-13 ALT cells to an overnight reaction with increasing amounts of Bal31. Restriction enzyme pre-digestion was done to facilitate Bal31 degradation. As shown in Figure 1a and Figure 1b, five units of Bal31 per reaction (0.5U/µg DNA) abolished the signal after hybridization with a telomere-specific probe. In order to address the question whether Bal31 digestion extends DNA degradation beyond telomeric sequences, 10µg of MCF-7 DNA was subjected to RsaI/HinfI digestion and a subsequent Bal31 reaction. DNA was separated on a agarose gel and hybridized with a probe targeting a microsatellite region. Whereas in samples without Bal31 treatment a smear of short fragments remained, samples having undergone Bal31 treatment showed no signal at a concentration of 0.125U Bal31/µg DNA (Figure 1c).

According to a model of t-circle generation in ALT cells proposed by Wang et al. one would expect most of the t-circles generated by a homologous recombination process contain nicks and gaps (Wang R. C. et al., 2004). Since Bal31 also has endonuclease activity and cleaves double-stranded DNA at nicks and gaps, we reasoned that filling these gaps with Klenow enzyme prior to Bal31 digestion would spare circular telomeric DNA that would otherwise be degraded by the enzyme. Ten µg of ALT cell VA-13 DNA was digested with RsaI and HinfI. The samples were then subjected to a Klenow fill-in reaction. Although Bal31 digests circular DNA with nicks at a much slower rate than it digests linear DNA, we tested whether an additional ligation step with T4 DNA ligase is required for DNA preservation (Przykorska A. K. et al., 1988). As shown in Figure 2a, only a faint signal was obtained for Bal31-degraded DNA after (TTAGGG)_n hybridization. However, Klenow-fill in prior to Bal31 treatment yielded a much stronger signal, whereas an additional ligation reaction did not improve signal intensity further. For telomerase-positive H1299 cells no signal was obtained. In order to determine whether the product generated by Klenow treatment is also resistant to other nucleases, we used T7-exonuclease or Mung Bean nuclease instead of Bal31, and obtained the same result (data not shown). We next investigated whether pre-incubation with T7 endonuclease can restore Bal31 sensitivity. T7 endonuclease is an enzyme that digests double-stranded DNA, which is not perfectly matched, for instance when cruciform structures or heteroduplex regions are present. As shown in Figure 2b, T7 endonuclease treatment after the Klenow procedure made DNA sensitive to Bal31 treatment.

In order to rule out the possibility that linear telomeric ends and all their associated structures are the source of the signal detected by Klenow/Bal31 treatment, we performed a telomeric separation experiment. Application of this technique permits double-stranded telomeric DNA with a G-rich overhang as well as G-rich single-stranded DNA to be separated from genomic and double-stranded circular DNA which remain in the supernatant fraction. In pre-experiments the efficacy of telomere retrieval was tested. We thereby compared the amount of telomeric DNA captured from 40µg of RsaI/HinfI-digested DNA with 20µg of supernatant DNA. By comparing signal intensity between the retrieved and the un-retrieved telomeric DNA we estimated a recovery rate of approximately 37% which is in good accordance with previously published data (Figure 3) (Wright W. E. et al., 1997). Klenow/Bal31 treatment of 40µg of supernatant DNA after telomeric separation yielded a signal in VA-13 ALT cells but not in SW39 telomerase-positive cells. In contrast, the Klenow/Bal31 procedure performed with the telomeres retrieved from 40µg of DNA showed no signal, namely neither in SW39 nor for VA-13 cells. This suggests that with linear telomeric DNA molecules no product can be generated with our Klenow/Bal31 t-circle detection procedure.

Klenow/Bal31 treatment amplifies telomeric DNA

In order to compare our method with a standard technique we did a 2D gel electrophoresis experiment (Figure 4). In RsaI/HinfI-digested DNA without Klenow/Bal31 manipulation a prominent arc representing the chromosomal telomeric DNA is detectable (indicated by a white arrow). Only in VA13 cells is a separate, less intense arc visible which indicates the presence of extrachromosomal telomeric DNA (black arrow). After Klenow/Bal31 treatment the genomic arc is absent in both cell lines. However in VA-13 cells the t-circle protocol generates DNA molecules that run noticeably different during 2D electrophoresis than the extra-chromosomal telomeric DNA of unmodified VA-13 DNA. It further shows that the signal obtained after enzymatic modification is considerably stronger than the signal from native extrachromosomal telomeric DNA. In order to further elucidate the migration pattern of the t-circle product a comigration experiment was performed. Genomic DNA from VA-13 cells was admixed with circular plasmid DNA containing an ampicillin resistance gene. After 2D-gel electrophoresis telomeric and plasmid DNA were visualized by hybridizing with a telomere-specific probe followed by stripping and re-hybridization with an ampicillin-specific probe. As expected, the circular plasmid DNA ran higher than did the t-circle product generated from VA-13 DNA, indicating that the Klenow/Bal31 treatment does not generate circular DNA (data not shown).

The T-circle product is specific for ALT cells

In order to address the specificity of our assay we tested our t-circle protocol in different cell lines using either the ALT or the telomerase pathway of telomere length control. As shown in Figure 5a, all ALT cell lines tested revealed the presence of a signal, whereas all telomerase-positive cells were negative. Signal intensity between the various cell lines tested was different, with VA-13 cells reproducibly showing a strong telomeric signal, whereas SW26 cells generally yielded lesser t-circle product. We further analyzed ALT cells with ectopically overexpressed hTERT. Telomerase activity in ALT cell lines is able to inhibit the ALT pathway, causing a loss of ALT-typical features (Ford L. P. et al., 2001). Figure 5b shows a TRF analysis of VA-13 cells and two clones, which have telomerase activity introduced. In these clones either elongation of the shortest telomeres occurred (VA-13 + hTERT#1) or overall telomere length was drastically reduced (VA-13 + hTERT#7). Application of our t-circle protocol to DNA samples from these cells shows a t-circle product only in VA-13 cells. Cells containing telomerase activity lacked a t-circle product irrespective of the telomere length in these cell lines (Figure 5b). In addition, DNA from normal human fibroblast (BJ cells) analyzed at various population doublings also did not

exhibit a t-circle product (data not shown). Taken together, this indicates that our protocol generates a product that occurs only in telomerase-negative ALT cells.

Discussion

Bal31 treatment of DNA that has been reduced to TRF size by RsaI/HinfI digestion efficiently degrades linear DNA molecules. As far as telomeric DNA is concerned a concentration of 0.5U Bal31 per μg DNA digests the overly long telomeres in ALT cells as efficiently as it digests the telomeres from telomerase-positive cells in an overnight reaction. A Klenow reaction prior to Bal31 digestion however generates a telomeric signal in ALT cells but not in telomerase-positive cells which is detectable in 1-D electrophoresis. Selective enrichment of linear telomeric DNA in ALT and telomerase positive cells did not result in a telomeric signal following our enzymatic treatment, suggesting that the circular molecules present in the total DNA extract give rise to the product generated with our t-circle protocol. Application of our assay to various ALT cell lines identified them as being positive for the t-circle product, whereas telomerase-positive cells were negative. Cell clones from an ALT cell line where the ALT pathway was blocked by telomerase expression became negative for the presence of a t-circle product.

In previous studies electron microscopy and 2-D gel electrophoresis have shown that t-circles correlate with the size of the loop portion of t-loops and are present only in ALT cells (Cesare A. J. et al., 2004). Although the precise mechanism responsible for generating these circles is still elusive it was recently shown that XRCC-3 and NBS-1, two proteins involved in DNA recombination are required for t-circle formation (Compton S. A. et al., 2007). Disruption of the shelterin complex by overexpression of a mutant allele of TRF-2 causes telomeric deletion events and the generation of t-circles. Again, this is a process that is dependent on XRCC-3 (Wang R. C. et al., 2004). The working model for t-circle generation is based on the concept that t-loops are resolved into t-circles via a homologous recombination process. The predicted product of such a recombination process is a relaxed circle containing nicks and gaps in both strands. Relaxed circles are in fact the predominant fraction of the extrachromosomal telomeric DNA detected in 2-D gel electrophoresis (Wang R. C. et al., 2004). The presence of small gaps, however, makes t-circles sensitive to Bal31 degradation. Klenow fill-in of gaps increases the fraction of Bal31-resistant DNA molecules, which therefore improves the sensitivity for detecting these circular structures.

Our protocol clearly shows that the telomeric signal is amplified following a Klenow reaction. The mechanism of this amplification process has not been addressed experimentally. However, we speculate that the strand displacement capabilities of the Klenow enzyme occurring at DNA nicks and small gaps might contribute to this (Walker G. T. et al., 1992). Once strands are displaced, the 3' end at the nick might initiate a rolling circle process that takes place on both strands, thereby generating double-stranded telomeric DNA (Figure 6). Hybridization of gels under non-denaturing conditions for the C- as well as for the G-strand did not give rise to any detectable signal, making the presence of significant amounts of single-stranded DNA unlikely (data not shown). Such a rolling circle mechanism might also explain the nature of the product generated with our protocol. 2-D gel electrophoresis clearly shows that what is generated by Klenow/Bal31 treatment is not circular DNA. The product possesses migration characteristics which mimic those of linear DNA molecules. One explanation is that the rolling circle mechanism stops when the nick or gap of the template strand is reached, which results in a linear molecule. Complex tertiary structures formed by pure TTAGGG sequences might attribute to this nuclease resistance. Using T7 endonuclease, which acts on heteroduplex DNA, cruciform or mismatched DNA structures after the Klenow reaction restored Bal31 sensitivity. This argues in favor of the idea that our t-circle protocol generates a linear, albeit complex, telomeric DNA molecule.

Taken together, these data suggest that although the t-circle product is not circular DNA its starting material must be of circular origin in order to generate a detectable product after enzymatic treatment.

A similar technique was recently published (Zellinger B. et al., 2007). Here a rolling circle amplification with ϕ 29 polymerase of denatured plant telomeric DNA hybridized to a complementary primer generates large single-stranded DNA products. Exonuclease V, which degrades linear double-stranded and single-stranded DNA from both ends applied prior to the polymerase reaction, confirms assay specificity for the detection of circular telomeric DNA. Like our approach, the product generated by this technique is not circular, although the molecules it aims to detect are of circular origin.

Comparison of various ALT cell lines with our t-circle protocol shows a marked difference in the signal intensity detected. Signal intensity of extrachromosomal telomeric DNA in SW26 cells was reproducible at a lower level than was that of VA-13 cells. Wang et al. compared telomeric circles in different ALT cell lines and found a strong signal in five out of six cell lines, whereas in one cell line only moderate intensity was detected (Wang R. C. et al., 2004). They also reported a difference in the presence of supercoiled circular DNA. These data suggest that in different ALT cell lines extrachromosomal telomeric DNA is composed of different types of molecules that are present in different quantities. The difference in signal intensity observed with our method could therefore be a reflection of the different amount of extrachromosomal circular telomeric DNA present in these ALT cell lines. However, this was not addressed in a quantitative manner.

Our current strategies for diagnosing the ALT pathway in tumors include the use of various diagnostic features. However, none of them is highly specific for ALT cells. This dilemma is due to our lack of understanding of the basic mechanism that engages this pathway. Hence, we depend on using a combination of good surrogate markers. The presence of telomeric circles is one feature of ALT cells, and techniques that enable their detection in an easy way are highly useful. Performing 1D gel electrophoresis instead of the more sophisticated 2D approach is of clear advantage. Our protocol allows the procedure to be done in a shorter time with a larger number of samples that can be run through the assay. Furthermore an amplification process as part of the procedure increases the sensitivity of a given method. The more accurate our tools are in detecting ALT-positive tumor cells, the more accurate we will be in distinguishing between telomerase-positive tumor areas and regions containing predominantly ALT cells. This would likely have a prognostic impact as it has been shown for liposarcomas recently (Costa A. et al., 2006). Moreover the ALT pathway can presumably serve as a mechanism of treatment resistance when telomerase activity is blocked. Circular telomeric DNA generated *via* a recombination process could initiate a roll and spread mechanism that leads to telomere elongation as it has been shown in *K. lactis* (Natarajan S. et al., 2002). Their detection could therefore be a sign for treatment failure. Since telomerase inhibitory therapies are on the verge of becoming a new tool in cancer treatment, the detection of such treatment-resistant cells via engagement of ALT is of the utmost clinical importance.

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Figure 1.

Bal31 degrades linear telomeric DNA. Ten μg of Hind III-digested genomic DNA from telomerase-positive MCF-7 cells (Figure 1a) and VA-13 ALT cells (Figure 1b) was subjected to increasing amounts of Bal31 in an overnight reaction (1.25U, 2.5U, 5U, and 10U of Bal31). Equal loading was assured by OD measurement of the DNA samples. After electrophoresis the gel was hybridized with a telomere-specific probe (TTAGGG)₄. Increasing amounts of Bal31 reduced the telomeric signal obtained, with 0.5U Bal31 per μg DNA being the amount that abolishes the detection of telomeric sequences. Complete degradation of genomic DNA was confirmed by hybridization with a probe targeting a microsatellite region (Figure 1c).

Figure 2.

Klenow treatment prior to Bal31 incubation preserves the telomeric signal. Bal31 degradation of 10 μ g of RsaI/HinfI-digested genomic DNA from VA-13 cells resulted in a faint signal after (TTAGGG)₄ hybridization. A Klenow fill-in reaction prior to Bal31 incubation drastically increased signal intensity. Additional ligation of DNA after fill-in was not associated with greater assay sensitivity. DNA from telomerase-positive H1299 cells did not give any signal (Figure 2a). Bal31 sensitivity of Klenow-treated DNA from VA-13 cells was restored by T7 endonuclease treatment prior to Bal31 incubation (Figure 2b).

Figure 3.

Klenow/Bal31 treatment does not generate a product from linear telomeric DNA. After digesting 40 μ g of genomic DNA with *RsaI* and *HinfI* a biotin-labeled C-rich oligo was annealed to the 3' single-stranded overhang. Pulling down oligo-bound DNA with streptavidin-coated magnetic beads allows separation of linear telomeric DNA from circular molecules. TRF analysis of the supernatant and pulled down DNA allows an estimation of the telomere retrieval efficiency. Subjecting DNA to a t-circle protocol showed that only in the supernatant fraction a detectable signal was obtained, whereas the pull-down fraction remained negative after Klenow/Bal31 treatment.

Figure 4.

The Klenow/Bal31 treatment of ALT cell DNA generates molecules that run as a single arc in 2D gel electrophoresis. 20 μ g of *RsaI*/*HinfI*-digested genomic DNA from telomerase-positive SW39 (upper part of the figure) and VA13 ALT cells (lower part of the figure) was subjected to a 2D gel electrophoresis and hybridized with a telomere-specific probe.

Samples without Klenow/Bal31 treatment showed the presence of chromosomal telomeric DNA in both cell lines (white arrow), whereas extrachromosomal telomeric DNA was detectable in the VA13 sample only (black arrow). As compared to SW39 cells the Klenow/Bal31 procedure generates a distinct single arc only for VA-13 DNA with a noticeably different migration pattern than that exhibited by the extrachromosomal telomeric DNA in the untreated VA-13 sample.

Figure 5.

T-circles are detected in ALT but are lacking in telomerase-positive cells. Various ALT cell lines tested positive for the presence of t-circles. In contrast, t-circles were absent in telomerase-positive cell lines (TA) (Figure 5a). ALT cell clones that have lost ALT-typical features due to overexpression of telomerase lack a detectable Klenow/Bal31 product (Figure 5b).

Figure 6.

A model by which strand displacement capabilities of the Klenow enzyme produce a linear t-circle product. At the 3' DNA end at nicks and gaps present in t-circles a rolling circle mechanism might be initiated following strand displacement. Both strands could be involved which would generate a linear double-stranded telomeric DNA molecule.