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A human interstitial telomere associates *in vivo* with specific TRF2 and TIN2 proteins

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Mammalian telomeres are composed of long arrays of TTAGGG repeats that form a nucleoprotein complex which protects the chromosome ends. Human telomere function is known to require two TTAGGG repeat factors, TRF1 and TRF2, and several interacting proteins, but the mechanism by which the DNA/protein complex prevents end to end fusion *in vivo* has not been elucidated. In order to better understand the role of specific telomere-associated proteins in the organisation of chromosome ends, we have studied a patient with a rare chromosome rearrangement that has given rise to an interstitial telomere. Using specific antibodies and immuno-FISH on unfixed metaphase chromosomes, we show that the proteins TRF2 and TIN2 (TIN2 interacts with TRF1) co-localise with the interstitial TTAGGG repeats. Our results demonstrate, for the first time in humans, that TRF2 and TIN2 proteins associate with interstitial duplex TTAGGG repeats, *in vivo*. They confirm that double stranded-telomeric repeats, even when complexed with specific proteins, are not sufficient to create a functional telomere. Finally, they suggest a possible role for proteins in stabilising interstitial TTAGGG repeats.
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Introduction

Telomeres are specialised DNA/protein structures that act as protective caps to prevent end fusion and to distinguish the chromosome ends from double-stranded breaks. Mammalian telomeric DNA is composed of several kilobases of duplex TTAGGG repeats terminating in a 3′ single-stranded G-rich DNA tail.[1]

Telomeric DNA repeats have been shown to be associated with the specific TTAGGG repeat factors TRF1 and TRF2. Both factors bind *in vitro* to the duplex DNA repeats with high affinity[2] and binding does not require a DNA end.[3,4] However, very low affinity binding to single-stranded DNA has recently been demonstrated,[5] with a net preference for the G-strand. TRF1 and TRF2 are found to co-localise, *in vivo*, with telomeres of human metaphase chromosomes.[2,6,7] From a functional point of view, both have been suggested to stimulate formation of the terminal telomeric t-loop, with TRF1 coiling the duplex telomeric tract back on itself, allowing TRF2 to promote the invasion of the single-stranded 3′ overhang into the duplex telomeric repeat array.[8] Moreover, TRF1 is thought to promote parallel pairing of telomeric DNA[2] and TRF2 to prevent chromosome fusion.[9]

Human telomeres contain other proteins that do not bind telomeric DNA directly and localize to telomeres via protein/protein interaction, as is the case for TIN2[10] and tankyrase[11] interacting with TRF1, and for hRap1[12] interacting with TRF2. Finally, although the Ku heterodimer is known to localise to telomeres via protein/protein interaction, it binds to DNA ends through its repair function, which renders possible its interaction with telomeric DNA.[13–15]

Nevertheless the mechanism by which the DNA/protein complex acts to prevent end-to-end fusion, *in vivo*, remains to be elucidated.

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In order to better understand the role of specific telomere-associated proteins in the organisation of chromosome ends, we tested for their presence at an interstitial telomere in a patient with a rearranged chromosome. This chromosome resulted from the translocation of an acentric chromosome 13 onto the short arm of an apparently intact X chromosome, creating a non functional interstitial telomere. In this study, using specific antibodies and immuno-FISH on unfixed metaphase chromosomes, we demonstrate, for the first time in humans, that the telomere-associated proteins TRF2 and TIN2 associate with interstitial TTAGGG repeats.

Material and methods

Cytogenetic analysis of the patient

The rearranged chromosome was fortuitously detected in a newborn referred for infectious disease, and presenting an isolated discrete lymphoedema of the feet, without any other evident clinical sign.

Routine chromosome analysis was performed on RHG-banded metaphase chromosomes obtained from PHA-stimulated peripheral blood lymphocytes, using standard protocols. In addition, the possibility that distal Xp sequences had been deleted from the rearranged chromosome was tested by fluorescence in situ hybridisation (FISH) with digoxigenin-labelled STS (steroid sulphatase) and KAL (Kalmann) cosmid probes (Oncor) and a biotin-labelled Xp/Yp-specific telomere probe (Clinisciences), all of which detect loci at the distal end of Xp.

Replication studies of the X chromosomes were performed using a very late terminal 5-bromo-2′-deoxyuridine (BrdU) pulse for the last 3 h of culture (60 μg/ml). BrdU incorporation was visualised using FITC-conjugated anti-BrdU antibody (Boehringer–Mannheim) as previously described.[16] The X chromosomes were identified by an X alphoid digoxigenin-labelled probe (Oncor), hybridised in situ to the preparations before the immunostaining protocol.

In order to search for telomeric TTAGGG repeats, a FITC-labelled PNA (CCCTAA)$_3$ probe (DAKO) was hybridised in situ to fixed metaphase chromosomes, as recommended by the supplier.

All preparations subjected to FISH were then counterstained with antifade (Vectashield, Vector Laboratories) containing 100 ng/ml DAPI (4,6-diamidino 2-phenyl indole) and analysed by fluorescence microscopy.

Specific antibodies

The presence of two telomere-specific proteins at the interstitial telomere was studied: the TTAGGG repeat factor TRF2 and the TRF1-interacting protein TIN2. TRF2 was detected with a rabbit polyclonal antibody raised against aminoacids AA306 to AA391 (kind gift from E Gilson and K Ancelin, ENS Lyon, France). TIN2 was detected using a rabbit polyclonal antibody raised against AA121 to AA354,[10] kindly provided by J Campisi and SH Kim (Berkeley, USA).

Immuno-FISH protocol

Unfixed chromosome preparations were obtained from an EBV-transformed lymphoblastoid cell line derived from the patient. After colchicin treatment, cells were incubated in hypotonic solution (diluted foetal bovine serum, 20%) for 10 min at 37°C. They were then collected by centrifugation and resuspended in fresh hypotonic solution, the cell density being adjusted to $10^4 - 10^5$ cells/ml. Cells were kept on ice and metaphase spreads were prepared within 1 h. Aliquots (0.5 ml) of cell suspension were centrifuged (300 t/min for 10 min) onto polylysine-coated slides in a cytospin 3 (Shandon). For the experiment with TIN2, the quality of spread preparations was improved by adding glycerol (0.5%) to the cell suspension before centrifugation. The metaphase preparations were then fixed at room temperature for 10 min in freshly prepared 4% (w/v) paraformaldehyde/PBS pH 7.2. Slides were then rinsed briefly in PBS and used immediately.

A permeabilisation step was performed with 0.1% Triton X-100 in PBS for 40 min at room temperature, followed by at least 15 min in blocking buffer (PBS/0.05% Triton X-100/5% goat serum/5% fœtal calf serum). Cell preparations were then incubated with buffer (PBS/0.05% Triton X-100) containing either anti-TRF2 diluted 1/1000, or anti-TIN2 diluted 1/50, for 2 h at room temperature. DNAse 1 (10 ng/ml) was added to the primary incubation mixture in order to improve antibody accessibility.[7] After washing (3 × 5 min) in PBS, primary antibodies were detected with a Cyanin 3-conjugated affinity purified goat anti-rabbit IgG (Tebu) diluted 1/300 in PBS/0.05% Triton X-100, for 1 h at room temperature. Preparations were then fixed with 4% (w/v) paraformaldehyde in PBS for 5 min and counterstained with DAPI (100 ng/ml) diluted in vectashield.

All antibodies were tested in individual staining reactions for their specificity and performance. Controls without primary antibodies were all negative. To exclude potential artifacts induced by FISH, the immunofluorescent signal given by each antibody on metaphase chromosomes was first analysed and photographed. The same preparations were subsequently subjected to the double colour FISH protocol using both a chromosome 13 digoxigenin-labelled paint probe and a chromosome X biotin-labelled paint probe (Oncor) and the signals detected as recommended by the manufacturer. Preparations were then counterstained with DAPI at 100 ng/ml in vectashield and re-photographed.

Microscopy analysis

Metaphase preparations were all observed using an Axioplan-2 Zeiss fluorescent microscope. The images were captured with a CCD camera (photometrics 'Sensys'). Information was collected and merged using IPLabs Spectrum software (Vysis).

Results

Characterisation of the karyotype of the patient

Standard banding techniques showed that the karyotype of the patient consisted of only 45 chromosomes, with an acentric chromosome 13 translocated onto the short arm of an apparently normal X chromosome. The formula was 45,X, − 13, − X,+derX t(X;13)(p22;q11). We detected no loss of X chromosome material on the rearranged der X, using either RHG-banding technique or FISH with various distal Xp telomeric probes (STS, KAL, and the Xp/Yp specific telomere)(data not shown).

Moreover, FISH with a specific FITC-labelled PNA telomere probe allowed us to identify interstitial, non-functional telomeric TTAGGG repeats on the derived chromosome, located close to the translocation junction (Figure 1). These interstitial telomeric repeats are presumably derived from the Xp telomere. In all metaphases analysed, the FISH-signal obtained at the interstitial site appeared very similar in intensity to the signals located at the extremities of the rearranged chromosome. Despite the presence of internal telomeric sequences, the rearranged chromosome appeared to be stable in 200 metaphases analysed.

Inactivation studies demonstrated that the translocated X chromosome was inactivated in 49/60 (81.6%) metaphases with detectable BrdU incorporation. However, no difference in BrdU incorporation was detected between the normal and
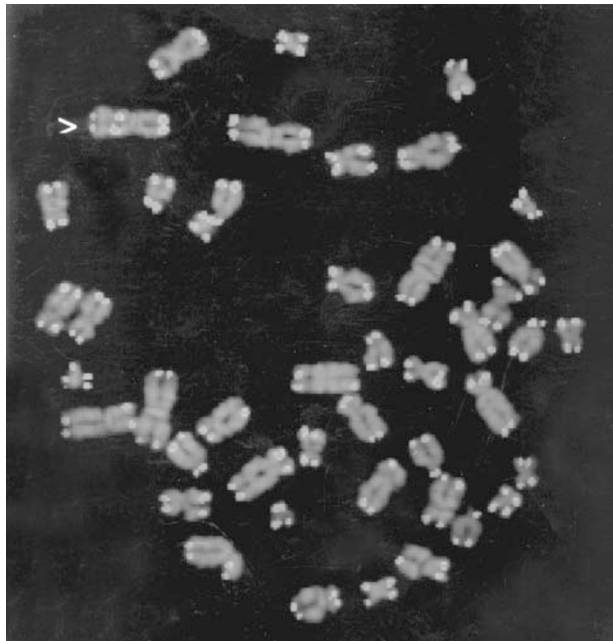
Figure 1 The TTAGGG repeats are detected by fluorescence *in situ* hybridisation on fixed metaphase chromosomes. FITC-labelled PNA (CCCTAA)$_3$ probe hybridised on metaphase of the patient shows fluorescent spots on the telomere ends of each chromosome and an interstitial signal on the rearranged chromosome.

the translocated chromosome 13 (data not shown), suggesting that inactivation had not spread to the translocated autosomal segment. This could result from the presence of interstitial repetitive DNA sequences, as was previously observed for a similar rearrangement involving chromosome 9 beta-satellite sequences.[17] It might also be the result of a limiting amount of Xi specific transcript (XIST) synthesised from the XIST gene on the inactive X chromosome. For the translocation product described here, XIST transcript level would then only be sufficient to inactivate the entire X chromosome, but not the adjacent chromosome 13.

Immunostaining results

Unfixed metaphases of the patient were subjected to immunostaining with two telomere-specific proteins, TRF2 and TIN2 (which interacts with TRF1). The rearranged chromosome was subsequently identified by double colour FISH performed on the same preparations.

Immunofluorescence performed with the anti-TRF2 polyclonal antibody revealed a punctate pattern in interphase nuclei and one or two spots at the ends of metaphase chromosomes. Moreover, on well spread metaphases, a clear interstitial protein signal was detected on a large chromosome. This chromosome was then identified as the translocation product by FISH, and the anti-TRF2 signal shown to be located at the junction between chromosome 13 and the chromosome X short arm (Figure 2).

In order to improve protein preservation during the spreading protocol, unfixed metaphase preparations were obtained with the addition of 0.5% glycerol to the cell suspension before the cytospin step. Under these conditions, the anti-TIN2 antibody resulted in a signal distribution on metaphase chromosome that was very similar to that obtained with anti-TRF2 antibody. In several metaphases, where the rearranged chromosome lay at the periphery, a distinct signal was visible at the interstitial telomere, demonstrating the presence of TIN2 protein and suggesting the presence of TRF1 protein at this site (Figure 3).[10]

Discussion and conclusion

In order to better understand the role of specific telomere-associated proteins in telomere organisation, we tested for their presence at an interstitial and non functional telomere in a patient with an (X;13) translocation. Using specific antibodies and immuno-FISH on unfixed metaphase chromosomes, we showed the co-localisation of telomeric DNA and the two telomeric proteins TRF2 and TIN2 at the translocation breakpoint.

We first showed that TRF2 associates with interstitial duplex TTAGGG repeats. TRF2 has been demonstrated to specifically bind duplex telomeric DNA sequences *in vitro* and to localize *in vivo* at the telomeres of interphase and metaphase chromosomes.[4,6,7] Nevertheless, electron microscopy studies have raised doubts as to whether TRF2 binds to
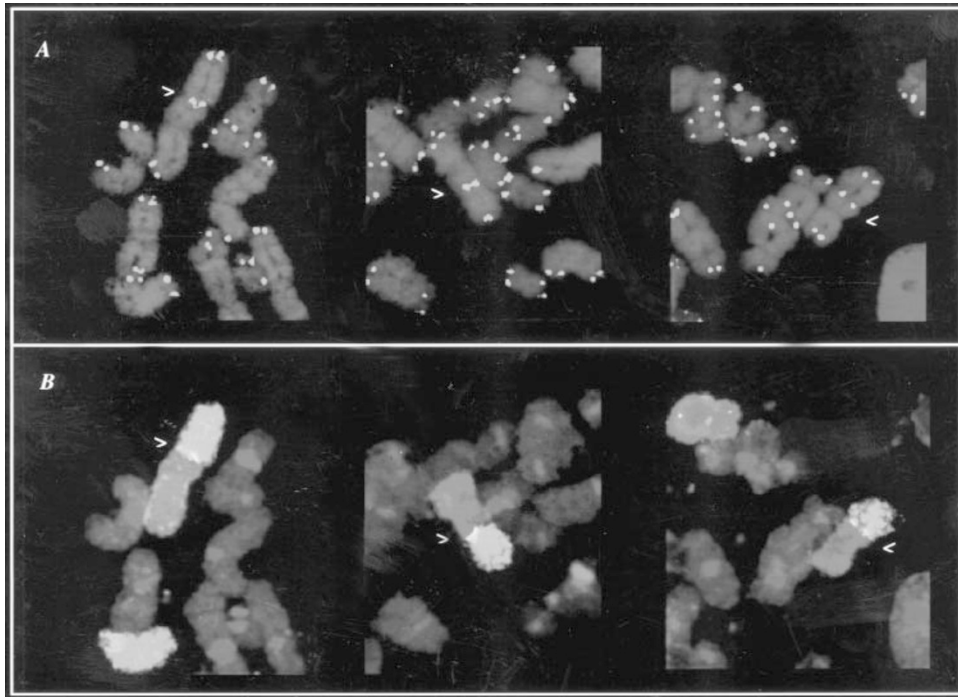
Figure 2 The TRF2 protein is present at the interstitial telomere of the rearranged chromosome (indicated by an arrowhead). (**A**) Three partial unfixed metaphases after immunostaining with anti-TRF2 specific polyclonal antibody. (**B**) The rearranged chromosome is subsequently identified by FISH using chromosome 13 and chromosome X paint probes.
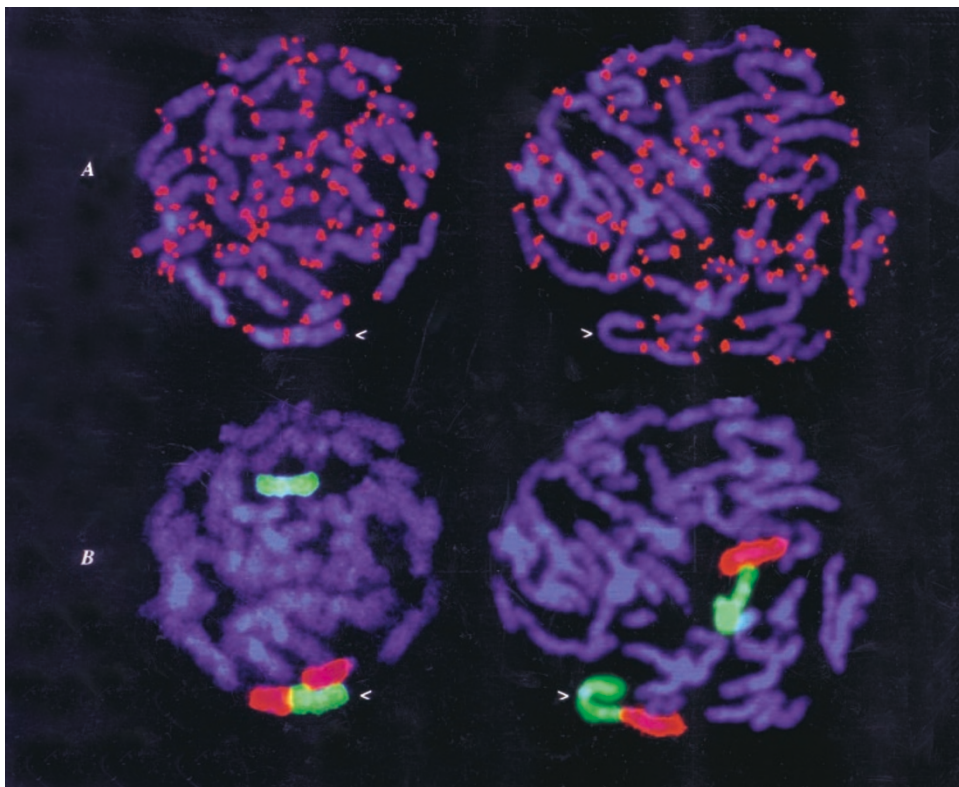
Figure 3 The TIN2 protein is present at the interstitial telomere of the rearranged chromosome (indicated by an arrowhead). (**A**) Two unfixed metaphases after immunostaining with anti-TIN2 specific polyclonal antibody. (**B**) The arranged chromosome is subsequently identified by FISH using chromosome 13 (red) and chromosome X (green) paint probes.

duplex telomeric DNA or to a particular structural feature of telomeres *in vivo*.[8,18] In the rearranged chromosome described here, the internal repeats do not appear to be organised as a functional telomere, and no features evocating a telomeric structure are observed. Moreover, it is reasonable to assume that no single-stranded DNA is present within the interstitial repeats, since if left unrepaired it would lead to broken chromatids detectable in metaphases. We observed no such instability in metaphases, and our results are therefore consistent with the hypothesis that TRF2 binds directly to human duplex telomeric DNA *in vivo*.

We next showed that TIN2 associates with interstitial TTAGGG repeats in metaphases. Nevertheless, TIN2 does not bind double-stranded TTAGGG repeats directly, and has been demonstrated to localise to telomeres through its interaction with TRF1.[10] Although we cannot exclude the intervention of other factors, our results suggest that TRF1 is present at the interstitial TTAGGG repeats. In agreement with this hypothesis, TRF1 has been demonstrated to colocalise with large interstitial TTAGGG repeats in immortal Chinese Hamster ovary cells.[19]

Our results therefore confirm that double-stranded telomeric repeats, even when complexed with specific telomere proteins which normally prevent chromosome fusion,[9] are not sufficient to create a functional telomere. In functional telomeres, a 3' single-stranded G-rich DNA tail has been demonstrated to form a t-loop *in vitro*, in the presence of both the TTAGGG double-stranded DNA, and the TRF2 protein.[8] On the basis of the lack of single-stranded DNA and the presence of double-stranded DNA and TRF2 at the site of interstitial telomere, our results highlight the role of the 3' single-stranded G-rich DNA tail in the organisation of a functional telomere. We suggest that it is the lack of a single-stranded DNA tail at the internal repeats that prevents the formation of a t-loop structure even in the presence of TRF2.

The internal telomeric TTAGGG repeat appears to be stable. Interstitial telomeric sequences are rarely found in human constitutional pathologies[20–26] although they are more commonly reported in tumoral pathologies.[27–29] In mammals, internal telomeric sequences have been shown to be hotspots for meiotic recombination.[30,31] Moreover, they are mitotically unstable being the site of breakage, fragility and jumping translocations which result from somatic recombination between the interstitial telomere and the telomeres of normal chromosomes.[32–34] It has also been shown that transfection of plasmids containing telomere repeat sequences results in chromosome breaks.[35,36] Strikingly, the interstitial repeats described here show great stability.

This stability could be the result of a very low copy number of the repeat sequence at the internal telomere, which might decrease the probability of somatic recombination events. However, the intensity of the FISH signal (by direct microscopic observation under non-saturated conditions)

did not appear significantly different at the internal telomere compared to the normal telomeres.

The stability of internal TTAGGG repeats, in this case, might also be linked to the late replication of the translocated (X;13) chromosome. As a result, the translocated chromosome could be isolated in an heterochromatic structure, that allows it to escape non homologous somatic recombination.

Finally, the stability of these internal repeats may also be conferred by the presence of specific telomere proteins, such as TRF2, TIN2 and possibly TRF1. TRF2 and TRF1 might recognise the interstitial TTAGGG repeats, with other proteins, such as TIN2, promoting a compact stable structure. In support to this idea TRF1 and TRF2, which bind to the stable interstitial TTAGGG blocks in immortal Chinese Hamster ovary cells,[18,19] have been suggested to promote stability by reducing unequal homologous recombination between TTAGGG sequences.[19]

In conclusion, the immuno-localisation of telomere-associated proteins in different pathological situations should help us to understand the organisation of the DNA/protein complex found at internal and distal telomere sites of chromosomes, and the mechanisms involved in telomere stabilisation, *in vivo*.

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