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Molecular basis of artifacts in the detection of telomerase activity and a modified primer for a more robust 'TRAP' assay

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ABSTRACT

Human somatic cells have essentially no telomerase activity. Telomerase is linked to tumor genesis and is a valuable marker for malignant growth. Extreme paucity of the enzyme neccessitated development of a PCR-based assay, 'telomeric repeat amplification protocol' (TRAP). Unfortunately, this method is not without difficulties. Amplification products are not related to the size of the amplified telomerase products. Furthermore, false positive results can occur, and careful control of reaction conditions is crucial. We analyzed in detail the molecular basis of artifacts. Based on these data, reverse PCR primer was changed and both problems in the TRAP assay were eliminated.

Telomerase is a ribonucleoprotein with the function of a DNA polymerase, where a segment of the RNA component functions as internal template. 3′-termini of linear chromosomes are the natural primers for telomerase. It adds telomer hexamer repeats, with substantial pausing after the addition of each hexamer. In vertebrates, the repeat sequence is TTAGGG (1). Telomerase activity is a potentially very useful tumor marker. A sensitive and reliable assay is of high interest, but it is hampered by the extreme paucity of the enzyme. For *in vitro* assays, synthetic non-telomeric oligonucleotides can be used as primers (2) and Kim *et al.* (3) developed the 'telomeric repeat amplification protocol' (TRAP), where telomerase products are amplified by a subsequent PCR (Fig. 1). Already in the presentation of the method, a peculiar artifact was described (3). Amplification of a well defined, synthetic telomerase product which contains four repeats (oligo TL) does not result in a single product of original size. Instead, a product ladder with 6 base pair steps extends up to the top of the gel. This is identical to the result observed in an authentic positive telomerase assay. Supposedly, this is caused by staggered annealing of CX primer (3) (see Figs 1 and 2A). Although T:T mismatches in primer CX reduced the artifact, an efficient suppression was not achieved.

We had the following apprehension: highly variable amounts of the tentative 'primer dimer' were observed (3,4). If primers CX and TS yield a real primer dimer, this is very similar to TL (Figs 1 and 2A). This can lead to false positive telomerase assays, since subsequent staggered annealing results in a hexamer ladder. Indeed, we observed large amounts of artifact products, if (i) the sensitivity of the assay was increased by high numbers of PCR cycles, or (ii) the wax barrier was eliminated to allow a simple set-up with a complete mastermix (Fig. 3A).

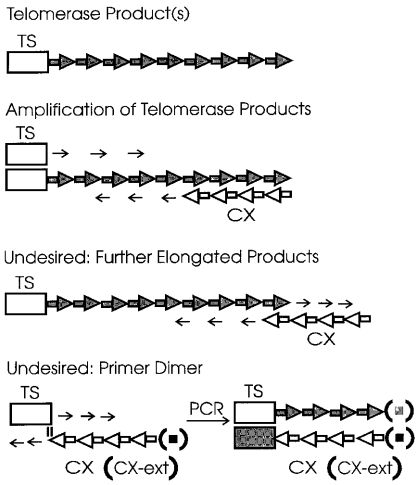

Figure 1. Scheme for PCR products in the TRAP assay. For sequences, see Figure 2. First, telomerase adds telomeric hexamer repeats (shaded arrows) onto primer TS (white square). In subsequent PCR (indicated by short arrows), excess primer TS is combined with reverse primer CX (four complementary telomeric repeats; white arrows). Undesired, further elongated products occur due to staggered annealing with one (or more) hexamer repeats. In spite of the minimal overlap (two A:T pairs), primer dimer formation yields elongated TS with four repeats, and as a real problem, staggered annealing results in a product ladder. Indicated in brackets: the extra 5′-terminal sequence in CX-ext (black square) prevents further elongation of dimer products.

Artifacts were analyzed in detail with 5′-^{32}P-labeled primers (Fig. 2C and D). Only the combination with labeled TS yielded a prominent artifact ladder, whereas it was much weaker with labeled CX. Chemical sequencing of the products confirmed dimer formation (as shown in Fig. 2A and D; further data not shown). Mismatches (introduced by the CX primer) reduced

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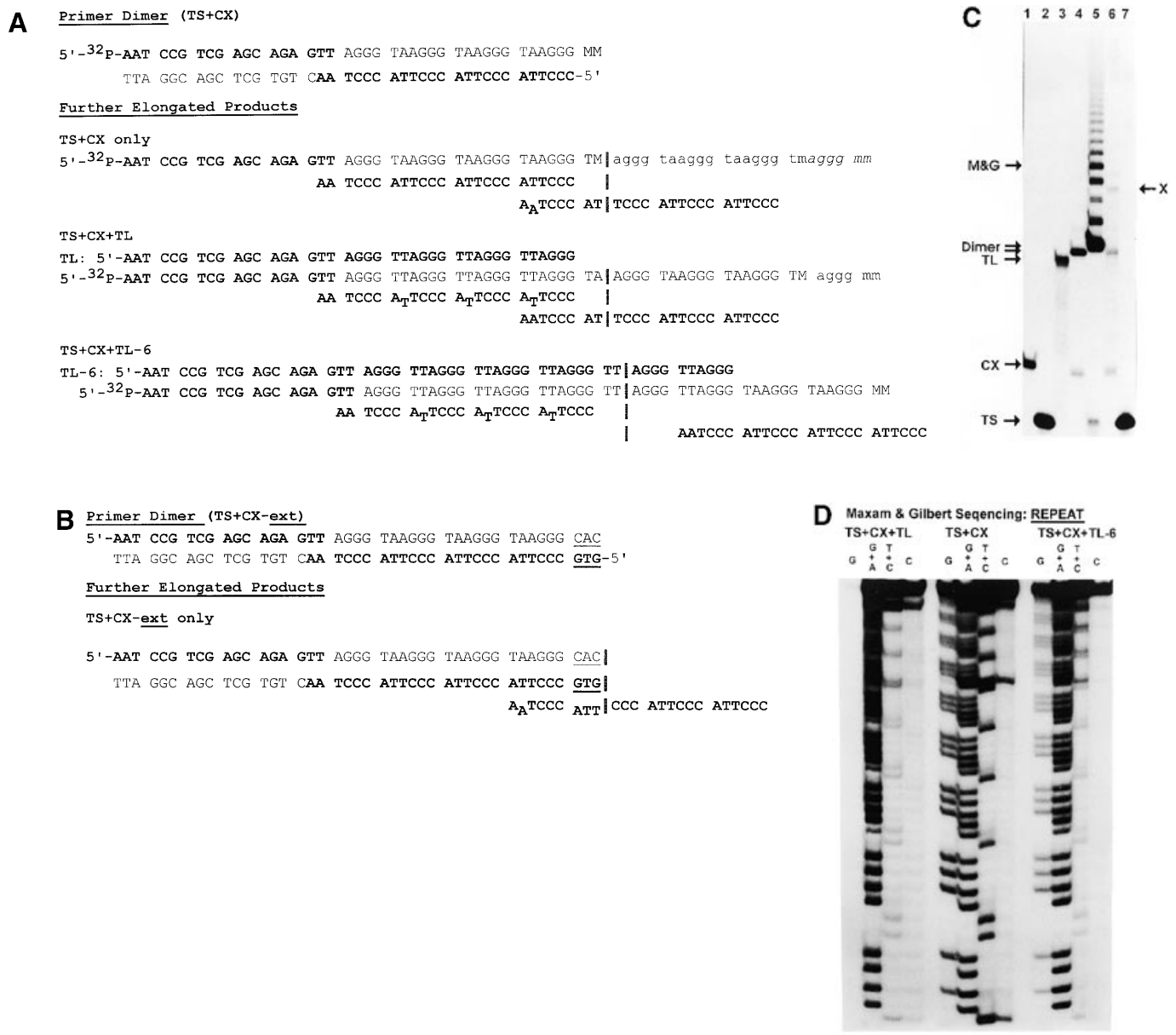

Figure 2. Sequences of PCR products in the TRAP assay. (**A**) Synthetic oligos are in bold. 5′-^{32}P-labeled sequences have been determined [example in (D)]. Indicated positions with M (=A,T,C) give prominent bands in the C-lane (D). Primer Dimer: here, elongation of both strands is shown (normal letters). TS+CX only: elongated TS has to accumulate in the dimer (TM position and vertical bar). Then, staggered annealing can occur with all four, three, two or (as shown here) with only one hexamer repeat of CX. Subsequent extension of TS is shown in small letters. Again, product accumulation precedes further extension (small letters in italics). A:A mismatch with CX is indicated. TS+CX+TL: no accumulation step is needed for staggered annealing with TL and CX. But further extension (small letters) requires accumulation (TM position). Internal T:T mismatches are indicated. TS+CX+TL-6, up to nine repeats can be added without accumulation step. (**B**) Primers TS and CX-ext. Elongated TS in the primer dimer has an extra 3′-terminal trinucleotide that essentially eliminates further extensions. (**C**) PCR products with 5′-^{32}P-labeled primers. Reactions were performed as described (3,4), but wax barrier and T4gp32 were omitted. Lanes 1–3, only one 5′-^{32}P-labeled primer was present. Lanes 4–7, labeled and unlabeled primers were combined: lane 4, 5′-^{32}P-CX and TS; lane 5, CX and 5′-^{32}P-TS; lane 6, 5′-^{32}P-CX and TL; lane 7, 5′-^{32}P-TS and TL. Positions of primers and the hypothetical dimer (complementary strands migrate slightly differently) are marked; X indicates an elongation product of primer CX (possibly staggered annealing with three hexamer repeats). (**D**) Elongation product (labeled M&G in panel C) and dimer band (not shown here) of 5′-^{32}P-labeled primer TS were isolated and analyzed by chemical sequencing (5). Base specificities of the sequencing reactions are indicated above the lanes (in the G-lane of TS+CX+TL most of the material was lost).

staggered annealing and elongation of CX, but not the formation of a product ladder with TS (Fig. 2A and C and data not shown). This means, unlike usual PCR artifacts, the observed artifact ladder is caused mostly by single-stranded products. Although this should limit amplification yields, here we have the unusual situation that the high excess of primer CX provides a template for essentially unlimited amplification.

Obviously, staggered annealing occurs in a stepwise fashion. Only three extra repeats can be added directly, and the corresponding product accumulates prior to subsequent staggering (Fig. 2A). An interesting observation is based on the ability of *Taq* DNA polymerase to add non-encoded 3′-terminal nucleotides (A, but also T and C). Accumulation of these intermediates is evident by introduction of a characteristic C-nucleotide (Fig. 2D). For

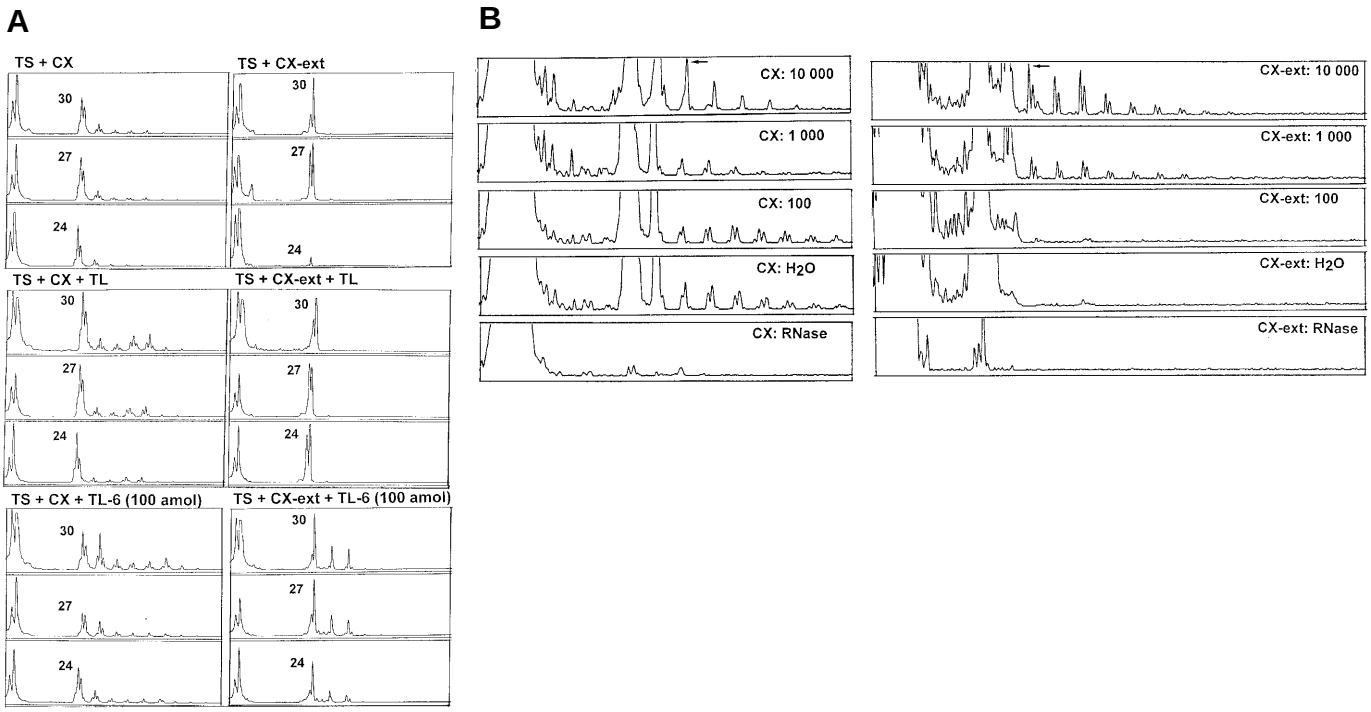

Figure 3. PCR product analysis with ABI prism 310 capillary electrophoresis unit. 5′-TAMRA-TS (NAPS GmbH, Göttingen) was used for labeling. PCR was performed by combining 48 µl of mastermix, containing all components [final 50 µl reaction volume: 20 mM Tris–HCl (pH 8), 50 µM dNTPs, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.005% Tween 20, 15 pmol primer TS and CX (or CX-ext) and 2 U *Taq* DNA polymerase (Appligene, Heidelberg)]. After adding 2 µl of the appropriate template oligo, PCR cycles were performed in an Eppendorf Mastercycler equipped with a hot lid: 30 s at 94°C, 30 s at 50°C, 30 s at 72°C (A); for telomerase assay (B), 2 µl of cell extract were added and incubated for 10 min at 30°C, before starting the PCR cycles. For sample analysis, 1 µl of PCR products were combined with 19 µl of formamide denaturation mix. **(A)** Three sets are shown for each primer combination, after 24 and up to 30 PCR cycles. Free TS migrates at the left margin, cycle numbers are left of the dimer position. Products with reverse primer CX display triplet bands: none, one or two extra nucleotides added beyond 3′-terminal GGG (Fig. 2A); doublet bands with CX-ext: only 1 extra nucleotide. 100 amoles of synthetic templates TL or TL-6 were used. **(B)** Assay of telomerase activity. Vertical scale was adjusted to product with two repeats beyond dimer (arrow). 27 PCR cycles were performed. Reverse primer and cell number [L428 cells (6)], or sample type are indicated (RNase: 10^4 cells inactivated with 100 ng RNase A).

TS+CX only, dimer products with four repeats accumulate before further extension by slippage can occur. This accumulation step corresponds to a prominent band in the otherwise blank C-lane. This indicates that 3′-elongation after staggered annealing preferentially occurred with a penultimate T and A,T or C as 3′-terminal nucleotide. Staggered annealing is limited again after three further repeats, with another band in the C-lane. The second accumulation step was also evident with TL. With TL-6 as positive control, a limit for slippage is expected only after nine repeats. This agrees with the absence of strong bands in the C-lane, since the analyzed product contained only eight repeats.

Considering the now well documented origin of artifacts, we designed an altered reverse primer CX-ext. Three additional 5′-terminal nucleotides limit staggered annealing. Thus, artifacts were essentially eliminated (Figs 1, 2B and 3A). An absolutely reliable recognition of telomerase positive samples is without problems, and with 33 PCR cycles the sensitivity could be increased to 30 cells (Fig. 3B; further data not shown). Furthermore, the maximum size of authentic telomerase products can be determined and assembly of a complete mastermix at room temperature allows a considerably simplified procedure.

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