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Telomere analysis by fluorescence *in situ* hybridization and flow cytometry

M. Hultdin, E. Grönlund, K.-F. Norrback, E. Eriksson-Lindström, T. Just[1] and G. Roos*

Department of Pathology, Umeå University, S-90187 Umeå, Sweden and [1]Department of Immunocytochemistry, DAKO A/S, DK-2600 Glostrup, Denmark

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ABSTRACT

Determination of telomere length is traditionally performed by Southern blotting and densitometry, giving a mean telomere restriction fragment (TRF) value for the total cell population studied. Fluorescence *in situ* hybridization (FISH) of telomere repeats has been used to calculate telomere length, a method called quantitative (Q)-FISH. We here present a quantitative flow cytometric approach, Q-FISH^{FCM}, for evaluation of telomere length distribution in individual cells based on *in situ* hybridization using a fluorescein-labeled peptide nucleic acid (PNA) $(CCCTAA)_3$ probe and DNA staining with propidium iodide. A simple and rapid protocol with results within 30 h was developed giving high reproducibility. One important feature of the protocol was the use of an internal cell line control, giving an automatic compensation for potential differences in the hybridization steps. This protocol was tested successfully on cell lines and clinical samples from bone marrow, blood, lymph nodes and tonsils. A significant correlation was found between Southern blotting and Q-FISH^{FCM} telomere length values (P = 0.002). The mean sub-telomeric DNA length of the tested cell lines and clinical samples was estimated to be 3.2 kbp. With the Q-FISH^{FCM} method the fluorescence signal could be determined in different cell cycle phases, indicating that in human cells the vast majority of telomeric DNA is replicated early in S phase.

INTRODUCTION

The study of telomere dynamics in human cells has been in focus since the first observations of telomere length reduction in tumors as well as in normal blood cells with increasing age (1,2), and especially so after the first reports on telomerase activity in human tumors *in vivo* (3,4). Thereafter, a large number of publications have shown that a majority of malignant tumors are telomerase-positive, indicating an active mechanism for telomere preservation (reviewed in 5). The telomere length of a cell population has traditionally been estimated by Southern blotting using enzymes with restriction sites in sub-telomeric DNA. The telomere restriction fragments (TRFs) obtained contain telomeric DNA of variable length and sub-telomeric DNA with a length of ~2.5–4 kb (reviewed in 6). With this approach, a close association between TRF length and expected lifespan was demonstrated for *in vitro* fibroblast cell cultures. This observation was one basis for the telomere hypothesis of aging (reviewed in 7), which was recently proven in transfection experiments leading to telomerase activity in normal fibroblasts, preserved telomeres and increased lifespan (8,9). No obvious relationship between TRF length and telomerase activity exists in cell lines or in clinical samples and permanently growing lines have highly variable telomere lengths. There are indications that both telomerase activity and TRF length can have a heterogeneous expression in individual cell lines during culture (10) and the mechanisms regulating telomerase levels are essentially unknown. Regarding telomere length maintenance, there is strong support for the importance of telomere binding protein(s) and specifically so for the double-stranded T_2AG_3-repeat-binding TRF1, which acts as a negative regulator (11). Furthermore, alternative mechanisms for telomere maintenance seem to exist and telomerase-negative cell lines and tumors sometimes exhibit unusually long telomeres (12). A proper characterization of the telomere status of tumors should therefore include determination of both telomerase activity and telomere length, which is especially important in future cases, for which anti-telomerase therapy will be an option.

TRF calculation by Southern blotting gives an estimation of the telomere lengths of all cells in a sample and is a robust, although time consuming, method. One disadvantage is the contribution of sub-telomeric DNA sequences, which may vary from case to case, making the calculation less accurate. With fluorescence *in situ* hybridization (FISH) direct labeling of the telomeric repeats is achieved and telomere length data obtained by FISH using a quantititative approach (Q-FISH) have correlated well with TRF values obtained by Southern blotting (13). One informative application of telomere Q-FISH was recently shown in telomerase-negative mice with a homozygous deletion of the gene for the telomerase RNA component (14). The Q-FISH technique is labor intensive, time consuming, needs metaphase spreads and the method is presently not suitable for routine purposes. Flow cytometry is a well-established method for rapid detection of fluorescence signals in individual cells in suspension which has been applied to FISH studies using chromosome-specific probes (15–18). Recently, flow cytometry using a fluorescently labeled peptide nucleic acid (PNA) probe was used to estimate the telomere length in individual cells (19,20). In the present study we have adopted this approach to develop an improved flow cytometric FISH technique that gives telomere length values within 30 h. The method employs an internal cell control to monitor the accuracy of the different steps in the procedure, from fixation to hybridization and DNA staining. Furthermore, the control cell population also serves as an internal telomere length standard, which makes it possible to compare

*To whom correspondence should be addressed. Tel: + 46 90 785 1801; Fax: + 46 90 785 2829; Email: goran.roos@pathol.umu.se

different samples with high precision. The technique was developed primarily for hematopoietic cells and was successfully tested on permanent cell lines and clinical samples. The telomere length in different phases of the cell cycle could be monitored, showing that the majority of telomeric repeats were duplicated during early to mid S phase.

MATERIALS AND METHODS

Materials

Seventeen established, hematopoietic cell lines and cell suspensions from six benign tonsils, six normal bone marrows, five lymphomas, five leukemias and one case with myelodysplastic syndrome type III were studied. The cell lines were 1301 (T cell lymphoblastic leukemia), 1301-U1 (a sub-line of 1301), 1301-U2 (a sub-line of 1301), CCRF-CEM (T cell lymphoblastic leukemia, parent line to 1301), Molt 4 (T cell lymphoblastic leukemia), Jurkat (T cell lymphoblastic leukemia), Raji (Burkitt's lymphoma), Daudi (Burkitt's lymphoma), CB-M1-Ral-Sto (B cell lymphoblastoid), U-937 (histiocytic lymphoma), BL-42 (Burkitt's lymphoma), K562-4 (erythroleukemia), DG-75 (lymphoma), M3 (B cell, malignant, not classified), U-266 (myeloma), HDLM-2 (Hodgkin's disease) and L428 (Hodgkin's disease).

For comparisons of Southern blotting and flow cytometric data cells were harvested and frozen in DMSO medium at –120°C for FISH staining and DNA histogram analysis and as cell pellets at –80°C for Southern blotting.

Peptide nucleic acid (PNA) synthesis and labeling

The $(CCCTAA)_3$ PNA probe was synthesized using the Expedite 8909 Nucleic Acid Synthesis System (PerSeptive Biosystems, Framingham, MA) and was labeled at both the N- and C-termini with lysine-(5(6)-carboxyfluorescein). The probe was purified by reverse phase HPLC at 50°C and characterized by MALDI-TOF MS on a Hewlett Packard G 2025 A mass spectrometer (Hewlett Packard, San Fernando, CA). The molecular weight was found to be within 0.1% of the calculated molecular weight.

Fluorescence *in situ* hybridization

The procedure detailed below was partly based on previously published FISH protocols (13,19,21). Fresh cell suspensions were washed in phosphate-buffered saline (PBS), centrifuged at 400 *g* for 5 min and resuspended in 1 ml PBS. Cells frozen in DMSO were rapidly thawed, washed three times and resuspended in 1 ml PBS. The cells were stained with 1 mg/ml Trypan blue and counted in a Bürker chamber. The cell suspensions were mixed 1:1 with 1301 cells and a total of 2×10^6 mixed cells were collected in 1.5 ml tubes. PBS was added and the tubes centrifuged at 4900 *g* for 30 s. The pellets were resuspended in 400 µl Fix & Perm Reagent A (Caltag Laboratories, Burlingame, CA) and incubated for 15 min at room temperature. An aliquot of 1 ml PBS was added to each tube and after centrifugation the pellets were resuspended in 400 µl Fix & Perm Reagent B. After incubation for 15 min the cells were washed twice in PBS. Thereafter, 5×10^5 cells were collected in new Eppendorf tubes, PBS was added and after centrifugation the supernatants were removed. The pellets were resuspended in a hybridization mixture containing 70% formamide (47671; Fluka BioChemika, Buchs, Switzerland), 1% Blocking Reagent (1096176; Boehringer Mannheim GmbH, Mannheim, Germany) and 4 nM fluorescein-$(CCCTAA)_3$-fluorescein PNA probe in 10 mM Tris, pH 7.2. After incubation for 10 min at room temperature the tubes were carefully vortexed and placed in a water bath at 87°C for 10 min with continuous shaking. The tubes were placed in the dark at room temperature overnight (15–20 h), whereafter the cells were centrifuged and incubated twice at room temperature for 10 min in 70% formamide, 0.1% BSA (B-8894; Sigma BioSciences, St Louis, MO) and 0.1% Tween 20 (P-9416; Sigma Chemical Co., St Louis, MO) in 10 mM Tris, pH 7.2. The cells were then resuspended in 1 ml 0.15 M NaCl, 0.1% BSA, 0.1% Tween 20 in 50 mM Tris, pH 7.5, and transferred to 5 ml tubes. An additional 3–4 ml 0.15 M NaCl, 0.1% BSA, 0.1% Tween 20 in 50 mM Tris, pH 7.5, were added and after 10 min at room temperature the tubes were centrifuged at 400 *g* for 5 min. The cells were resuspended in 0.5 ml PBS and 20–30 µl were used for cytocentrifugation onto glass slides and covered by Mounting Medium (1000-4; Sigma Diagnostics, St Louis, MO) containing 0.025–0.1 µg/ml propidium iodide and 2.5% DABCO (D-2522; Sigma Chemical Co.). Propidium iodide was added to the remaining suspensions in a final concentration of 0.1 µg/ml, vortexed and then kept in the dark at 4°C for at least 30 min until flow cytometric analysis.

Flow cytometry

The analysis was performed in a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) using the FL1 channel for detection of fluorescein signal and the FL3 channel for propidium iodide. No compensation was set on the instrument. List mode data from 10^4 cells in each experiment were collected and analyzed using CELL-Quest software (Becton Dickinson). The telomere fluorescence signal was defined as the mean fluorescence signal in G_0/G_1 cells after subtraction of the background fluorescence signal (i.e. FISH procedure without probe). The relative telomere length value was calculated as the ratio between the telomere signal of each sample and the control cell line (1301) with compensation for the DNA index of G_0/G_1 cells. This compensation was performed in order to 'normalize' the number of telomere ends per cell. The complete procedure for telomere length estimation by FISH and flow cytometry as described above was denoted Q-FISH^{FCM}. The DNA index was estimated separately for each individual sample after propidium iodide staining according to Vindeløv *et al.* (22) using chicken and trout erythrocytes as internal controls. In all experiments described, the same batch of frozen 1301 cells in DMSO was used.

Confocal microscopy

Cytospin preparations were analyzed using a Sarastro 2000 CLSM confocal microscope (Molecular Dynamics, Sunnyvale, CA) equipped with an argon/krypton laser and ImageSpace software (Molecular Dynamics). Scan sections of 0.6 µm were studied using appropriate filters for simultaneous fluorescein and propidium iodide detection.

Southern blot analysis

Total DNA was extracted from fresh frozen cells or tissue pieces. Five micrograms of DNA were digested overnight with 5 U/µg *Hin*fI under conditions recommended by the manufacturer (Boehringer Mannheim GmbH). The DNA fragments were separated by electrophoresis through 0.5 or 0.7% agarose gels in 50 mM TBE buffer. The separated DNA fragments were

depurinated in 0.25 N HCl for 15 min, denatured in alkali and transferred to nylon membranes (Hybond-N; Amersham Life Science Ltd, Aylesbury, UK) using 20× SSC. Oligonucleotide probe $(TTAGGG)_4$ (Scandinavian Gene Synthesis AB, Köping, Sweden) was 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Amersham Life Science Ltd). Prehybridization and hybridization were performed at 48°C in QuikHyb Hybridization Solution (Stratagene, La Jolla, CA) using a thermostat controlled hybridization incubator (Techne, Cambridge, UK). Washes were performed according to the manufacturer's recommendations (Stratagene). Filters were autoradiographed (Hyperfilm-MP; Amersham Life Science Ltd) with an intensifying screen at –70°C for 12–48 h.

Densitometry and evaluation of the length of telomere fragments

Autoradiographs were scanned with a densitometer (Personal Densitometer; Molecular Dynamics). Mean telomere restriction fragment length (TRF) was defined as $(OD_i)/(OD_i/L_i)$, where OD_i is the densitometer output and L_i is the length of DNA at position i. Sums were calculated over the range 2–26 kb. The size of the DNA fragments in each lane was calculated in Excel® according to a previously described procedure (23). Telomere peak values were measured by estimating the band size corresponding to the point with the highest optical density within the peak.

RESULTS

Probe and cell concentrations

In the initial hybridizations we used a probe concentration of 0.3 µg/ml (55 nM), as described in previous studies (13,21), giving bright staining of the telomere ends under the fluorescence microscope and a flow cytometry signal easily discriminated from the control. The use of an internal cell line control (1301 cells) made possible further investigation of the optimal hybridization conditions for flow cytometric evaluation. The 1301 cell line is near-tetraploid and has long telomeres (>25 kb), which makes 1301 cells easy to identify in flow cytometric dot plots regarding both DNA content and telomere signals. For these reasons the 1301 cell line constituted an ideal internal control suitable for mixing with other cell populations. At optimal resolution the signal ratio (i.e. the difference in mean fluorescence channels) between 1301 cells and test cells should be maximal and by testing probe concentrations of from 5 pM to 550 nM we found that an optimal signal ratio was achieved at 2–6 nM PNA probe, as illustrated in Figures 1 and 2A and B. At higher probe concentrations the fluorescence signal was brighter under the microscope as well as in the flow cytometer, but, due to the accompanying higher background fluorescence, the signal ratio was increasingly impaired (Figs 1 and 2B).

Since the probe concentration was critical for optimal signal resolution, it was expected that cell concentration influenced the fluorescence signal. At a probe concentration of 4 nM stable signal ratios were obtained for cell concentrations between 2.5 and 7.5 × 10^5 cells in hybridization solution, but outside these values the signal ratio decreased. In all future hybridizations 4 nM probe and 5 × 10^5 cells were used to give optimal and reproducible results. With confocal microscopy distinct fluorescence signals with low backgrounds were obtained and examples of Q-FISH^{FCM}/DNA dot plots are shown for two cell lines and two patient samples in Figure 3.

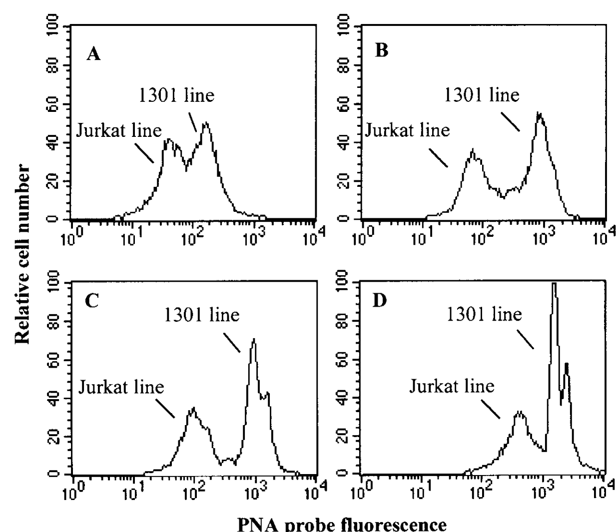

Figure 1. Cellular fluorescence detected by flow cytometry after hybridization of 1301 and Jurkat cells with the fluorescein-labeled PNA probe illustrating the effect of different probe concentrations. (**A**) 0.5 nM PNA probe; (**B**) 2.5 nM PNA probe; (**C**) 5 nM PNA probe; (**D**) 50 nM PNA probe.

Since 1301 cells were added in each experiment prior to fixation and hybridization, errors due to variations in the hybridization and DNA staining conditions were minimized. Using the procedures detailed above, single cells were maintained in suspension and a cell recovery of at least 50% was regularly achieved. On comparing the DNA histograms obtained by the FISH protocol with standard DNA histograms obtained by separate propidium iodide staining, we found that no selective loss of cells was observed during the Q-FISH^{FCM} procedure.

A correct fluorescence level was achieved by subtracting the autofluorescence of each cell population, detected in parallel runs with no probe added, from the fluorescence value obtained with the telomere probe. In the experiments we observed rather large variations in autofluorescence signals between individual cell lines and patient samples. Repeat experiments using 1301 cells and the CB-M1-Ral-Sto line gave a mean Q-FISH^{FCM} value for the CB-M1-Ral-Sto line of 0.083 ± 0.005 (n = 9). Analysis of variance from parallel, doublet staining of 52 samples gave a standard deviation of 0.00495. No obvious connection existed for the difference between paired samples and fluorescence level.

The number of chromosomes, and thus telomere ends, is strongly correlated with the DNA index calculated from DNA histogram analyses (24,25). Thus, for an accurate determination of the telomere signal, the FISH fluorescence detected in the flow cytometer had to be corrected accordingly, which means that for a diploid population the signal ratio between these cells and the tetraploid 1301 cells was multiplied by 2 (DNA indices 1 and 2 respectively).

Regarding propidium iodide staining, it was observed that incubation for at least 3 h before analysis was necessary in order to obtain acceptable DNA histograms. The fluorescein signal from the T_2AG_3 repeats could, however, be properly evaluated after 30 min. The time of analysis after adding propidium iodide was not critical and in one experiment six cell lines and patient samples were tested at four different time points (after 0, 4, 11 and 25 days), demonstrating a very low variation in telomere signal ratios, but the DNA histograms improved with prolonged incubation (not shown).

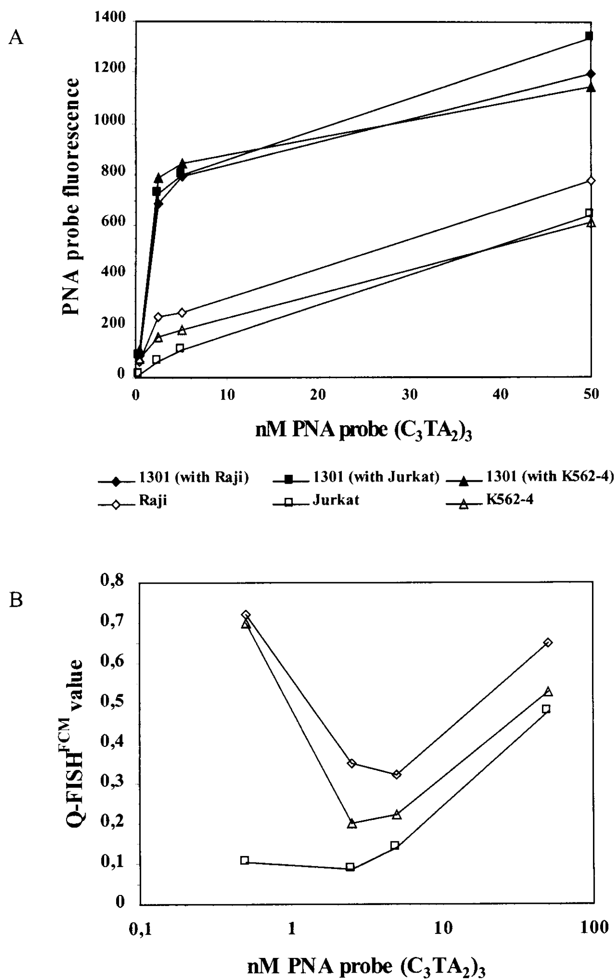

Figure 2. Fluorescence detected by flow cytometry after PNA probe hybridization of 1301 cells mixed with Raji, Jurkat or K562-4 cells. (**A**) At probe concentrations between 0.5 and 2.5 nM a rapid increase in fluorescence was detected and above 5 nM the increase was dependent on the background fluorescence, reflected as a parallel increase in signal intensity. The PNA probe fluorescence is denoted as channel number. (**B**) This figure illustrates the effect on Q-FISH^{FCM} values, i.e. the fluorescence ratio between the tested cell line and 1301 cells after subtraction of background fluorescence and after compensation for DNA ploidy, at probe concentrations between 0.5 and 50 nM (for details see Material and Methods).

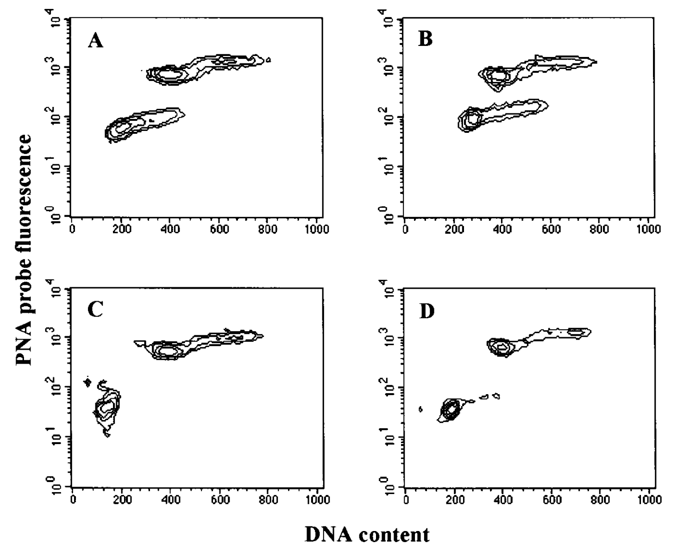

Figure 3. Contour plots showing PNA probe fluorescence versus DNA content in: (**A**) Jurkat and 1301 cells; (**B**) U937 and 1301 cells; (**C**) benign tonsil (sample L81) and 1301 cells; (**D**) acute myeloid leukemia type M5 (sample B200) and 1301 cells.

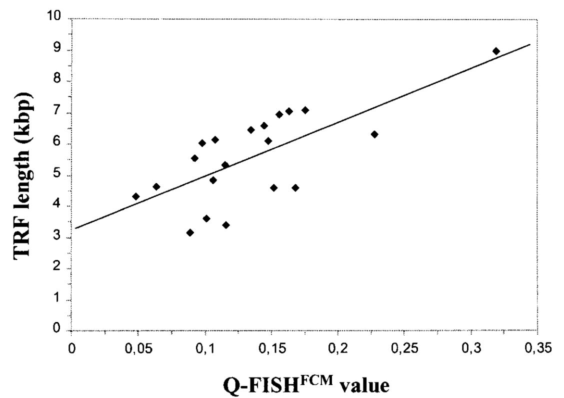

Figure 4. Correlation between telomere restriction fragment (TRF) length estimated by Southern blotting and Q-FISH^{FCM} values, showing a highly significant correlation ($P = 0.002$, $r^2 = 0.51$).

Telomere Q-FISH^{FCM} compared with Southern blotting

DNA extracted from 10 cell lines and 10 patient samples, benign as well as malignant, were subjected to Southern blotting using the $(T_2AG_3)_4$ probe and the telomere lengths were compared with the Q-FISH^{FCM} data obtained from the same samples. A highly significant correlation ($P = 0.002$, according to the Spearman test) existed between values achieved with the two techniques, as demonstrated in Figure 4. By extrapolation of the regression line a Q-FISH^{FCM} value of 0 corresponded to 3.2 kb in the Southern blot, a value which represented a mean of the sub-telomeric DNA lengths present in the samples tested (Fig. 4).

Cell lines

In the 17 tested cell lines telomere Q-FISH^{FCM} values between 0.08 and 1.0 were obtained. Interestingly, three different sub-lines (1301, 1301-U1 and 1301-U2) derived from the original CCRF-CEM line were investigated. The 1301-U1 and 1301-U2 lines were found ‘accidently’ when a large series of cell lines were checked for telomere lengths and analyzed by DNA fingerprint analysis using Southern blotting in 1994. The fingerprint results were confirmed in the present study using a $(CAC)_5$ probe (not shown). The ‘parent’ CCRF-CEM line was diploid and exhibited short telomeres, in contrast to 1301, which was near-tetraploid with very long telomeres. The 1301-U1 sub-line was diploid but had long telomeres of similar lengths as in 1301 cells. 1301-U2 harbored both diploid and tetraploid cells and both populations demonstrated long telomeres. The data indicated that development of sub-line 1301 was accompanied by a considerable lengthening of the telomeres and in further sub-cultures of tetraploid as well as diploid cells, the long telomeres were stably maintained.

Patient samples

All patient samples were successfully analyzed showing Q-FISH^{FCM} values between 0.05 and 0.18. The samples were

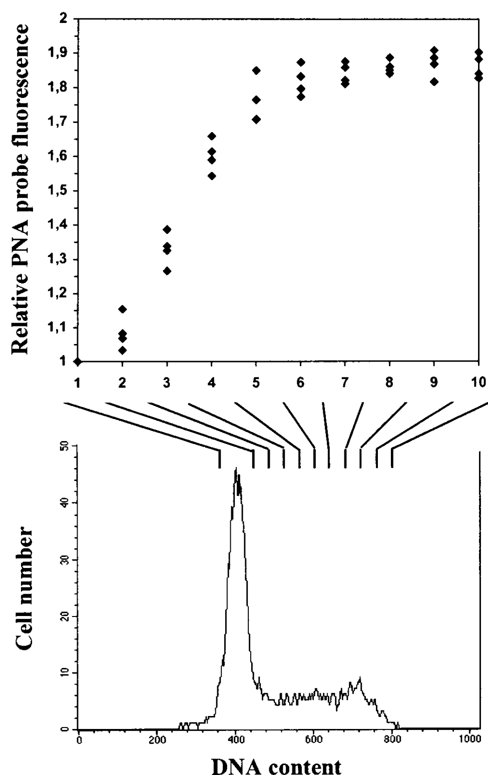

Figure 5. Relative fluorescein signal generated by the PNA probe in different cell cycle compartments defined by their DNA content as indicated. The data from four different experiments using 1301 cells are shown. The PNA probe fluorescence signal had increased by ~80% in mid S phase.

sub-grouped according to morphological diagnosis in one malignant and one benign group. The malignant samples contained in all cases a dominant neoplastic cell population. Somewhat lower Q-FISH^{FCM} values were obtained in the malignant compared with the benign samples (mean values 0.08 and 0.13 respectively). The samples were used primarily for evaluation of the Q-FISH^{FCM} technique and no further analysis was performed.

Telomere replication

The conditions used for the Q-FISH^{FCM} analysis were not optimal for DNA staining, although rather good quality DNA histograms were obtained, especially after prolonged incubation. DNA histograms of good quality could be used for calculation of the FISH signal in separate sub-populations defined by their DNA content, as illustrated in Figure 5. This analysis was performed on four separate hybridizations of 1301 cells and we found that the telomere signal consistently increased considerably faster than the DNA content and, in fact, the major part of the telomere sequences were replicated during the first half of S phase (Fig. 5). During the whole S phase the telomeric signal increased 80–90% using the evaluation procedure defined. Similar results were obtained in four patient samples, two of which were malignant (acute myeloid leukemia and T cell lymphoblastic lymphoma) and two of which represented benign bone marrows.

DISCUSSION

The Q-FISH^{FCM} approach described in the present study offers a convenient and robust technique for telomere length estimation on cells in suspension and determination of telomere repeats per cell can be performed on thousands of cells within seconds. Using this method, cycling as well as non-cycling cells can be studied and no biased selection of cells occurs from fixation to final analysis. These features make it the method of choice for characterization of telomere status and, since as few washing steps as possible were used, cell loss was comparably small (<50%). However, for measurement of single chromosome telomeres the quantitative microscopic technique is so far unchallenged.

Two features are of utmost importance for the accuracy of telomere length estimation using Q-FISH^{FCM}: (i) use of a PNA probe (19,20); (ii) inclusion of an internal standard control. In our first tests we used DNA probes with poor results, but when the fluorescein-labeled PNA probe was introduced into the system, a highly significant difference in fluorescence intensity was observed. Hybridization with the PNA probe was very consistent and extremely stable, as demonstrated in various experiments, including analysis of the same samples up to 25 days after the hybridization procedure. Also, a high specificity of the telomere PNA probe has been previously demonstrated (26).

The internal cell line control was essential in optimizing the methodology, since it made measurement of the resolution of the fluorescence signals possible, demonstrating that the optimal probe concentration was about 10 times less than previously used in FISH studies (13,21). In the initial experiments both RNase and protease treatments (19) were included, giving an increased cell loss but no obvious difference in fluorescence signals in comparison with tests where these steps were omitted. When the ideal conditions had been found, control cells were used as an internal standard and thereby effects of possible differences in the conditions in individual tubes were minimized and the data from different samples could reliably be compared. These features are not possible without using an internal standard. The 1301 cell line was used for this purpose, since it is tetraploid with unusually long telomeres and only exceptional human samples would be expected to overlap 1301 cells in both DNA content and telomere length. One alternative to 1301 or similar human cells is mouse cells displaying telomeres >20 kb and we have used mouse spleen cells for this purpose without any problems.

The DNA histograms obtained with the Q-FISH^{FCM} procedure were of fairly good quality, but for proper ploidy evaluation DNA histograms achieved by standard staining of fresh, unfixed cells were needed. All samples to be tested by Q-FISH^{FCM} should therefore be analyzed separately for DNA ploidy in order to be able to make an accurate compensation for the cellular DNA content, which has previously been shown to correlate well with the number of chromosomes and thus with the number of telomeres (24,25).

The data obtained by Q-FISH^{FCM} showed a statistically significant correlation with telomere length determined by Southern blotting and densitometry. For Southern blotting, restriction enzymes are used which cut DNA in the sub-telomeric region and the telomere fragments detected with the T_2AG_3 probe thus contain non-telomeric DNA. With the Q-FISH^{FCM} method, telomeric repeats are labeled, but also potential intrachromosomal T_2AG_3 repeats will be included in the fluorescence signal. In the literature the prevalence of non-telomeric T_2AG_3 sequences in human chromosomes vary (27–30), but were found to be few and short using a sensitive PNA probe-based microscopic technique (26).

Thus, when comparing Southern blot data with Q-FISH^{FCM} values, the calculated regression line should cross the Southern blot axis at a point roughly corresponding to the mean sub-telomeric DNA length of the tested samples. This was the case, giving a value of ~3.2 kb, in good agreement with the 2.5–4 kb mean length of non-telomeric sequences estimated for human cells (reviewed in 6). This concordance using different approaches supports the idea that intrachromosomal T_2AG_3 repeats are of less significance for the total hybridization signal detected by flow cytometry.

Long-term cultured lines showed highly variable telomere lengths, whereas benign patient samples exhibited somewhat longer telomeres than malignant cases. The patient samples were primarily used for evaluation of the Q-FISH^{FCM} procedure and in order to properly evaluate whether telomere length is associated with morphological diagnosis, clinical parameters and outcome, larger patient samples are needed from different diagnostic sub-groups. The technique presented here will facilitate such future investigations.

One interesting result was obtained regarding the timing of telomere replication during cell cycle progress. For eukaryotes most studies have been performed on yeast cells, showing that telomeres can delay activation of replication origins until late S phase (31 and references therein) and this late replication program was established between mitosis and START in the subsequent G_1 phase (32). Few studies have focused on telomere replication and the cell cycle in human cells, indicating that telomeres can be replicated during the entire S phase and not only in late S phase, as predicted for most heterochromatic DNA structures (33). We could demonstrate that telomeric repeats were duplicated faster than bulk DNA and the vast majority of telomeric DNA was synthesized during the first half of S phase, which supports and extends previous observations. Future studies on various cell types, benign and malignant, will elucidate if this is a general feature of human cells. In our calculations the telomeric repeat fluorescence increased by 80–90% from G_1 to G_2/M and not by 100% as expected. This might be due to the fact that the G_1 gate used as a reference can harbor some S phase cells which might have started to replicate their telomeres. A non-linear relationship between the FISH signal and the number of telomeres cannot, however, be excluded and studies have been initiated to clarify this issue. Even with this in mind, the Q-FISH^{FCM} method provides a rapid and sensitive approach for quantitative analysis of repetitive DNA sequences in relation to the cell cycle.

In summary, the procedure presented here for flow cytometric evaluation of telomere length in individual cells offers an attractive alternative to hitherto established techniques and will significantly benefit future studies of cell lines and patient samples. Using flow cytometry, multiparameter analysis can be performed and Q-FISH^{FCM} staining can theoretically be combined with other fluorochromes coupled to various PNA probes or antibodies giving exciting possibilities for future refined analyses.

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