Amplification of competitive telomere sequence in living animal cells induces chromatin instability

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Abstract

We have reported the establishment of new episomal-type expression vector the copy number of which can be readily regulated by a temperature shift. In this study, we attempt to apply this vector for the functional analysis of the noncoding regions of DNA. A plasmid containing a 0.45 kb-telomere repeat sequence was constructed and transfected into simian CV-1 cells, leading to successful establishment of cell lines in which episomal telomere sequence could be amplified by temperature shift. When the episomal telomere sequence was amplified, the cells stopped proliferating at the G₂/M phase of the cell cycle and exhibited a large size with flattened morphology and several small nucleus-like particles. These cells expressed Cdk inhibitor p21 and β -galactosidase, which are expressed in some senescent cells. Microscopic analysis revealed frequent end-to-end attachments of chromosomes, which resulted in a variety of aberrant chromosome configurations. None of these characteristics was observed in nontransfected and control plasmid-transfected CV-1 cells at any cultivation temperature. These results indicate the usefulness of our vector system in analyzing telomeric DNA.

Abbreviations: Cdk, cyclin dependent kinase; SV40, simian virus 40.

Introduction

In eukaryotes, various cellular activities, such as regulation of cell growth and transcription or chromosome stability, depend on the appropriate functioning of the noncoding regions of DNA (Stief et al., 1989; Ikeno et al., 1998; Van Steensel et al., 1998). Such regions include telomeres that are located at the end of chromosomes, centromeres that mediate chromosome segregation during mitosis and matrix-associated regions that anchor each chromosomal DNA to nuclear scaffolds. We may be able to use these regions of DNA in recombinant DNA technology, when their special features have been elucidated: for example, matrix-associated regions greatly enhanced the expression of adjacent genes (Bode et al., 1992), and are therefore useful for constructing vectors with a high ability for

expression of foreign genes. However, the biological functions of most noncoding regions of DNA have not been elucidated yet because of the unavailability of satisfactory analytical methods especially for mammalian cells. In this study, we attempt to apply a new method for investigating the function of telomere.

Telomere is the unique sequence existing at the end of each linear chromosome in eukaryotes. Mammalian telomeres consist of long repeated sequences of a TTAGGG with single-stranded protrusion of a G-rich strand at their very end. Telomeric repeats were slightly shortened by each DNA replication, which is well-known as the 'replication end problem'. Thus, it is proposed that the telomere length plays a critical role in determining the residual proliferating capacity of the cells: when the telomere length becomes shorter than a hypothesized limit after repeated cell division, the cells stop growing and exhibit some specific morphological and biochemical features of what is referred to as cellular senescence (Hayflick, 1965;

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Harley et al., 1990; Noda et al., 1994; Dimri et al., 1995). The unlimited proliferative capacity of stem cells and tumors is maintained by telomerase, a recently cloned reverse transcriptase that continuously adds telomere repeats to the end of chromosomes using an essential RNA subunit as a template (Feng et al., 1995; Nakayama et al., 1997; Meyerson et al., 1997). Recent studies demonstrate that telomerase contributes to the stability of chromosomes by preventing aberrant fusions (Cenci et al., 1997; Kirk et al., 1997; Van Steensel et al., 1998). Linear DNA lacking telomere sequence aberrantly fused with other chromosomes, leading to a cycle of aberrant fusion and chromosome break called bridge-breakage-fusion cycle. Chromosomes were fused at their ends in cells lacking functional telomerases by inhibition of one of the telomere-binding proteins TRF2 (Van Steensel et al., 1998).

In previous studies, we have reported the establishment of a new episomal-type expression vector for mammalian cells (Kirinaka et al., 1994a, 1994b, 1995). The vector was based on the temperature-sensitive mutant of simian virus 40 (SV40) and its copy number could be readily regulated by mild change of cultivation temperature. We report here the application of this vector for functional analysis of the telomere region of DNA.

Materials and methods

Plasmids

Runaway-type plasmid vector pSVtsA was prepared by standard methods as previously described (Kirinaka et al., 1994a, 1994b). The plasmid pSVtsT containing telomere repeat sequence was constructed by inserting telomere repeat sequence of the plasmid pHTC into pSVtsA at the *Xho*I site as follows. pHTC is a plasmid containing telomere repeat sequence ((TTAGGG)_n, 450 bp in length) into the vector pTZ19U at the *Eco*RI site, and was kindly provided by Professor F. Ishikawa, Tokyo Institute of Technology. Telomere repeat sequence and minimal flanking sequences of pHTC were amplified by polymerase chain reaction with primers containing a *Xho*I recognition sequence, and subcloned into the *Xho*I site of pBluescript. After confirming the sequence of the insert, the telomere insert was excised and subcloned into the *Xho*I site of pSVtsA. Figure 1 shows the structure of the telomere-containing plasmid pSVtsT.

Figure 1. The structure of SV40-based episomal plasmid containing the telomere repeat sequence (pSVtsT). This plasmid has a temperature-sensitive (ts) large T antigen and a 450bp TTAGGG repeated sequence.

Establishment of telomere-transfected clones

CV-1 cells were transfected with plasmid DNA using the calcium phosphate coprecipitation method and propagated in a selective medium containing 500 μ g/ml of G418 (Life Technologies Inc., MD) as described (Kirinaka et al., 1994a), and the proliferating clones were isolated at 37 °C.

Synthetic telomere oligonucleotide ((TTAGGG)₅, purchased from Hokkaido System Science, Sapporo, Japan) and a large T-containing DNA fragment of SV40 (2.2 kb *Bam*HI-*Bst*XI fragment of pSVtsA, Kirinaka et al., 1994a) were labeled with ³²P and used as probes. The low-molecular weight DNAs were isolated and analyzed by dot blotting or Southern blotting as described (Kirinaka et al., 1994a).

Assessment of the characteristics of the established clones

The β -galactosidase was stained as described by Dimri et al. (1995). The amount of p21, one of the cyclin dependent kinase (Cdk) inhibitors, was assessed using anti-p21 polyclonal antibody (#sc-756, Santa Cruz Biotechnology, USA). To analyze the chromosomes at metaphase of the cell cycle, colcemid-treated cells were fixed, stained with Giemsa's solution, and

Figure 2. Regulation of copy number of telomere-containing plasmid by temperature shift. Low-molecular weight DNAs from either telomere-transfected clones (T1, T2) or vector-transfected clones (C1, C2) were analyzed by dot blotting using the large T antigen probe (A), and Southern blotting using the synthetic telomere oligonucleotide probe (B). Plasmid pSVtsT was also loaded to indicate the expected size in (B).

analyzed by microscopy as described (Van Steensel et al., 1998). All experiments were repeated at least twice to confirm reproducibility.

Results

Establishment of cell lines in which copy number of episomal telomere sequence can be regulated by temperature shift

In previous studies, we have reported the establishment of a new episomal-type expression vector for mammalian cells (Kirinaka et al., 1994a). The copy number of this vector could be readily regulated by a mild change of cultivation temperature: the number is below 100 copies per cell at the nonpermissive temperature of 39 °C, while it is over 5000 copies per cell at the permissive temperature of 33 °C. We intend to apply this vector for functional analysis of telomere. We expect that amplification of double-stranded telomere sequence deprives chromosomes of telomere-binding proteins, causing phenotypic changes in host cells.

CV-1 cells, in which the vector has been shown to replicate effectively (Kirinaka et al., 1994a), were

transfected with either telomere-containing pSVtsT or control pSVtsA plasmids. We investigated the differences in the copy number of the plasmid per cell when incubated under permissive (33 °C), semipermissive (37 °C) and nonpermissive temperatures (39 °C). The cells were incubated at the respective temperatures, and low-molecular weight DNAs were analyzed by the dot blot technique. As shown in Figure 2A, the average number of plasmid molecules was estimated to be around 2×10^2 copies per cell at 37 °C. The copy number of plasmids per cell was markedly higher, up to 1×10^4 at 33 °C. At the nonpermissive temperature of 39 °C, pSVtsA and pSVtsT plasmids scarcely replicated and the number of plasmids was around 50 copies per cell.

To confirm that episomal telomere sequence was not deleted in telomere-transfected clones, low-molecular weight DNAs were subjected to Southern blot analysis using a synthetic telomere probe. As shown in Figure 2B, telomere-transfected clones showed a band with an expected molecular weight of 0.45 kb, when the DNAs were digested by *XhoI* that excised the telomere inserts. Further analyses with *EcoRV*, which cut the plasmid at a single site, yielded

Figure 3. Morphological change and quantification of cell size in telomere-transfected clone. (A-F) Morphology of the cells. Cells of nontransfected CV-1 (A and B), vector-transfected (C and D) and telomere-transfected (E and F) clones were cultured for 6 days at the nonpermissive temperature of 39 °C (A, C and E) or at the permissive temperature of 33 °C (B, D and F). (G-J) Cell size analysis by EPICS XL. Each cell is plotted according to forward angle light scatter (indicating size) and side scatter (indicating vacuolization). Vector-transfected and telomere-transfected cells were cultured either at 39 °C (G, I) or 33 °C (H, J) for 6 days.

a band with an expected molecular weight of 9.5 kb.

Amplification of episomal telomere sequence induced morphological change of the cells

The morphology of the cells is shown in Figure 3. Nontransfected CV-1 and vector-transfected clones exhibited fibroblast-like shape independent of cultivation temperature (Fig. 3A-D). On the other hand, many cells of the telomere-transfected clones were large and exhibited flattened shape at 33°C (Fig. 3F) but not at 39°C (Fig. 3E). Some cells were 5 times as large as normal cells. To estimate the cell size quantitatively, the cells were analyzed with flow cytometer, by assessing forward angle light scatter, which indicates the size of each cell (Fig. 3G-J). When the vector-transfected CV-1 cells were cultured at 39 °C, the cells were small and the percentage of large cells that fell into the indicated gate was only 6% of the total population (Fig. 3G). The same was the case with the cells cultured at 33°C, the percentage of large cells being 10% (Fig. 3H). Similarly, in nontransfected cells, the percentages of large cells at 39 °C and 33 °C were 12%

and 11%, respectively (data not shown). On the other hand, the telomere-transfected clones contained a considerable percentage of large cells: when these cells were cultured at 39 °C, the percentage of large cells was 20% (Fig. 3I), which increased up to 34% at 33 °C (Fig. 3J). These results indicate that the size of the cells increased significantly when episomal telomere DNA was amplified in the living cells.

Amplification of episomal telomere sequence induced growth retardation of the cells at G₂/M phase

The enlarged cells were viable at least for 7 days in culture, judging from the exclusion of trypan blue, however, they ceased to proliferate. We next examined the cell growth rate of each clone by directly counting the viable cell number (Fig. 4A). At the nonpermissive temperature of 39 °C, both vector- and telomere-transfected clones rapidly proliferated, but the growth rate was slightly lower in the telomere-transfected clones. When the cells were cultured at 33 °C, vector-transfected clones proliferated rapidly at a rate comparable to nontransfected CV-1. On the

Figure 4. Growth rate and cell cycle analysis of telomere-transfected clone. Each clone was cultured for the indicated period, and viable cell number was counted by the trypan blue exclusion method (A). (C1: vector-transfected clone; T1: telomere-transfected clone) After 6 days of culture, cell cycle analysis using EPICS XL was performed by observation of the fluorescence of DNA-binding propidium iodide (B).

other hand, the telomere-transfected clones proliferated very slowly at 33 °C: their cell number was about one-eighth of that in the control clones after 260 h of culture, at which time their proliferative activity was almost lost.

Cell cycle distribution was examined by flow cytometry (Fig. 4B). When vector-transfected cells were cultured at 39 °C, 46% of cells were in G₀/G₁ phase, 23% in S phase and 24% in G₂/M phase. At 33 °C, 57%, 15% and 14% of the cells could be assigned to G₀/G₁, S and G₂/M phase, respectively. The results for nontransfected cells were quite similar (data not shown). When telomere-transfected clones were cultured at 39 °C, 36%, 18% and 14% of the cells could be assigned to G₀/G₁, S and G₂/M phase, respectively, not quite different from nontransfected and vector-transfected clones. However, when telomere-transfected clones were cultured at the permissive temperature of 33 °C, the percentages of G₀/G₁, S and G₂/M phase were 25%, 14% and 33%, respec-

tively, indicating the great increase in the number of cells in the G₂/M phase. In addition, small nuclei were detected. These results indicate that amplification of the episomal telomere sequence induced growth arrest of the cells at the G₂/M phase.

Amplification of episomal telomere sequence led to the expression of β -galactosidase and Cdk inhibitor p21

The expression of endogenous β -galactosidase was examined. The cells were cultured under each temperature condition for 6 days and the activity of β -galactosidase was assessed at pH 6.0. Neither nontransfected CV-1 (data not shown) nor vector-transfected clones (Fig. 5A, B) exhibited β -galactosidase activity under any culture condition. On the other hand, telomere-transfected clones exhibited significant β -galactosidase activity at 33 °C (Fig. 5D), which was absent at 39 °C (Fig. 5C). Moderate activ-

Figure 5. β -galactosidase staining. Vector-transfected clone at 39 °C (A) and 33 °C (B) or telomere-transfected clone at 39 °C (C) and 33 °C (D) were stained with X-gal to assess the β -galactosidase activity.

ity was detected in the telomere-transfected clones cultured at 37 °C (data not shown).

The amount of p21, one of the Cdk inhibitors that inhibit a wide variety of Cdks, was assessed by immunoblotting. As shown in Figure 6, faint bands were detected in vector-transfected clones independent of the cultivation temperature. Telomere-transfected clones expressed a similar amount of p21 at 39 °C, however, the amount was markedly higher in cells when cells were cultured at 33 °C. These results indicate that amplification of the episomal telomere sequence in the cells increased the amount of β -galactosidase and p21.

Amplification of episomal telomere sequence induced end-to-end fusion of chromosomes

We next analyzed chromosomes in metaphase (Fig. 7A). The cells were cultured for 2 days under several conditions and culture was continued for 16 h in the presence of 0.1 μ g/ml of colcemid, followed by standard chromosome analysis by Giemsa staining. When telomere-transfected clones were cultured

at 33 °C, we observed frequent end-to-end attachment that resulted in a variety of aberrant chromosome configurations such as ring chromosome or linear chromosome carrying multiple centromeres, commonly called chromosome train (d-f). On the other hand, most vector-transfected cells (a and b) and nontransfected cells (data not shown) did not contain such fused chromosomes. Telomere-transfected cells cultured at 39 °C also showed few aberrant chromosomes (c). Up to 39% of the telomere-transfected cells that were induced into metaphase at 33 °C had fused chromosomes, while only 8% of the cells had fused chromosomes at 39 °C (Fig. 7B). In vector-transfected clones and nontransfected CV-1 cells, the percentages of cells that had fused chromosomes were less than 6% at any cultivation temperature.

Discussion

In this study, we tried to apply a temperature-regulated episomal vector for the analysis of a noncoding region of DNA. We have succeeded in the establishment

Figure 6. Induction of Cdk inhibitor p21 in telomere-transfected clones. Cells of vector-transfected clone (C1) or telomere-transfected clone (T1) were cultured as described in the legend for Figure 3 and nuclear extracts were subjected to Western blotting to detect p21.

of clones having the telomere-containing plasmid pS-VtsT. These clones did not proliferate at the permissive temperature of 33 °C, at which the episomal plasmid was vigorously amplified.

We have obtained clear phenotypic changes by temperature shift of CV-1 cells transfected with the telomere repeat sequence. We consider that these were the results of an increased number of telomere repeat sequence in the cells because of following reasons. Vector-transfected cells did not exhibit such phenotypic changes whether cultured at nonpermissive or permissive temperatures. In addition, we have obtained some clones from telomere-transfected CV-1 cultures that did not show such changes. In these cells, the copy number of the episomal vector was increased

as expected, however, the telomere repeat sequence was deleted (data not shown). Cells that had episomal vector containing a 0.9 kb of erythropoietin gene did not exhibit such phenotypic changes (Kirinaka et al., 1994a and data not shown), excluding the possibility of the difference in plasmid size being the reason for such phenotypic changes. The mean telomere length varies in each cell line, and that of CV-1 has not been reported. We determined it by a standard procedure to be about 3 kb (our unpublished observation), estimating the total length of telomere repeat DNA per cell to be 360 kb. We consider that the amplified episomal telomere sequence, a total of 1×10^4 copies of 0.45 kb, about 12 times as long as the endogenous telomere, was sufficient to compete with endogenous telomere repeat sequence.

The phenotypes of telomere-amplified cells exhibited three major characteristics. The first is end-to-end fusion of chromosomes. The second is the growth arrest of the cells at G₂/M phase. The third is expression of β -galactosidase and Cdk inhibitor p21.

When telomere-transfected clones were cultured at the permissive temperature of 33 °C, we could readily observe end-to-end fusion of chromosomes in M phase nuclei. In accordance with our study, recent reports indicated that inhibiting the function of telomere prevented chromosome segregation and caused similar end-to-end fusion in various organisms (Cenci et al., 1997; Kirk et al., 1997; Van Steensel et al., 1998). These indicate the important role of telomere in normal chromosome segregation and in chromosome stability.

When the episomal telomere sequence was amplified, most of the cells were arrested at the G₂/M phase of the cell cycle. We consider that this growth arrest was caused by the abnormal chromosome segregation. This is supported by the fact that inhibition of chromosome segregation by colcemid leads to the growth arrest at G₂/M phase of the cell cycle. In addition, telomere-transfected cells at the permissive temperature had small nucleus-like particles that resembled the micronucleus induced by colcemid (our unpublished observation). Alternatively, increased expression of p21, one of the strongest natural inhibitors of G₁/S transition, might arrest the growth of the cells. However, since the cells were arrested at G₂/M phase in our study, it may be possible that increase in p21 level was not the reason for the growth arrest but rather a reflection of the altered gene expression induced by the change of telomere structure. From these results, we infer that an increased number of telomere se-

Figure 7. Instability of chromosomes in telomere-transfected clones. (A) Chromosome analysis at metaphase. Normal chromosomes in a vector-transfected clone at 39 °C (a) or 33 °C (b). Typical chromosomes in a telomere-transfected clone cultured at 39 °C (c). Aberrant chromosomes in a telomere-transfected clone at 33 °C: chromosomes with end-to-end fusion (d), chromosome rings with double telomere association (e), and chromosome train (f). Arrows indicate aberrant chromosome attachments. (B) Frequency of the cells with aberrant chromosome attachments in each clone was analyzed. At least 100 cells at metaphase in each clone were investigated. (C1,C2: vector-transfected clones; T1,T2: telomere-transfected clones).

quence inhibited normal segregation of chromosomes and arrested the cell cycle at M phase.

It is postulated that the length of telomeres is closely related to cellular senescence (Bodnar et al., 1998), which is characterized by some features including expression of β -galactosidase and Cdk inhibitor p21 (Dimri et al., 1995; Noda et al., 1994). In senescent cells, the change of gene expression was possibly caused by decrease in telomere length. In our study, the growth-arrested cells expressed β -galactosidase and p21. We can not estimate the length of cellular telomere in telomere-transfected clones because of large amount of episomal telomere sequence. Therefore, we can not determine whether the expression of these genes in our study was related to cellular senescence at present. The expression of p21 has been reported to be mediated by either p53-dependent or independent pathways (El-Deiry et al., 1993; Parker et al., 1995). In our case, large T proteins that were used for the replication of vector DNA bound and inactivated p53, excluding the possibility of p53-dependent transcription of p21.

We consider that the various phenotypic changes

induced by the amplification of episomal telomere sequence were caused owing to CV-1 cell chromosomes being deprived of telomere-binding proteins. Since our SV40-based plasmid had only a double-stranded telomere sequence, it could effectively deprive double-stranded chromosomal telomeres of binding proteins but not single-stranded telomere ends. To date, two proteins have been reported to specifically bind to mammalian double-stranded telomere DNA: TRF1 and TRF2. TRF1 is thought to be a negative regulator of telomere length because inhibition by the dominant negative form of TRF1 led to telomere elongation (Van Steensel and de Lange, 1997). TRF2 protects human telomeres from end-to-end fusion of chromosomes. Van Steensel et al. (1998) indicated that overexpression of dominant negative TRF2 induced end-to-end fusion of chromosomes. In addition, they also showed that inhibition of TRF2 induced a growth arrest with senescence-like characteristics: cells expressed β -galactosidase, became enlarged and had vacuolated cytoplasms with multiple small nuclei (Van Steensel et al., 1998). We obtained results similar to those reported by Van Steensel et al. (1998). Although

our vector system probably deprived chromosomes of at least TRF1 (and other unknown telomere-binding proteins) as well as TRF2, it is likely that the functional lack of TRF2 induced the most phenotypic changes in short term culture.

In our experimental system, cells exhibited clear phenotypic changes after very short periods of culture. We believe that the clones established in this study will be a convenient tool to study the mechanism of maintaining telomere and that this approach is useful for the analysis of other noncoding regions of DNA.

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