

Cytogenetic Studies

RADIATION-INDUCED MISREJOINED BREAKPOINTS IN HUMAN CHROMOSOMES: RANDOM OR NON-RANDOM?

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We investigated whether radiation-induced misrejoined chromosome breakpoints are randomly or non-randomly distributed throughout the human genome. Data were combined from as many published cytogenetic studies as possible. The percentage of radiation-induced breaks per megabase (Mb) of DNA between all human chromosomes was calculated, and the observed and expected numbers of breakpoints based on DNA content between and within chromosomes were compared. A DNA-proportional distribution of breakpoints in 14 autosomes and a statistically significant deviation from proportionality in the other 8 autosomes and the sex chromosomes was found. Regression analysis showed no significant change in breakpoint frequency per Mb of DNA relative to autosome size. Analysis between chromosome arms showed a non-random distribution of induced breakpoints within certain autosomes, particularly the acrocentrics. In cases of non-random distributions, a prevalence of events was found at heterochromatic regions and/or telomeres, and a clustering of breakpoints was found near the centromeres of many chromosomes. These results show that there is an approximately linear proportionality between autosomal DNA content and observed breakpoint number, suggesting that subsets of autosomes can be used to estimate accurately the overall genomic frequency of misrejoined breakpoints contingent upon a carefully selected subset. However, this conclusion may not apply to the sex chromosomes. The results also support the influence of chromatin organization and/or preferential DNA repair/misrejoining on the distribution of induced breakpoints. However, these effects are not sufficient at a global level to dismiss the value of cytogenetic analysis using a genome subset for biodosimetry.

CHROMOSOME ABERRATIONS OF CLONAL ORIGIN IN IRRADIATED AND UNEXPOSED INDIVIDUALS: ASSESSMENT AND IMPLICATIONS

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Chromosome painting has proven very useful for the detection of chromosomal rearrangements, although the presence of cells containing clonal aberrations can have an effect on the outcome of cytogenetic analyses (e.g. aberration frequency and chromosomal distribution studies). Cells with clonal chromosomal changes have been found from studies of both radiation-exposed Chernobyl liquidators and healthy unexposed human subjects. A detailed analysis of cytogenetic damage including identification of clones may prove useful for detecting stable rearrangements associated with cancer, since chromosome abnormalities have been associated with many human malignancies. We have utilized a simple statistical method to aid in the identification of subjects from distinct exposed and unexposed populations that may possess cells containing clonal rearrangements. A chi-square value determined from the observed number of aberrations and the expected number based on chromosome length that corresponds to a probability < 0.005 appears to be an indicator of clonality. These selected subjects can be analyzed further for clonality, thereby sparing detailed examination of the entire population. Necessary adjustments can then be made to prevent the overestimation of chromosome aberration frequencies. Here, we present an analysis of subjects possessing clonal aberrations to assess the influence of clonality on cytogenetic study results. The frequency of clonal aberrations appears to increase as a function of age in control subjects, whereas an age effect was not evident in radiation-exposed subjects. This suggests that spontaneous and radiation-induced clonal expansion are occurring in control subjects and liquidators, respectively. The presence of clonal aberrations in both control and Chernobyl radiation exposed individuals may be an important indicator of genomic instability and, ultimately, of the potential for malignant transformation.

LARGE-SCALE CLUSTERING OF RADIATION-INDUCED BREAKS ALONG CHROMOSOMES

David J. Brenner (with Rainer K. Sachs [UC Berkeley], Philip J. Hahnfeldt and Lynn R. Hlatky [Harvard Medical School]).

We have analyzed intrachromosomal clustering of DSBs (DNA double strand breaks) induced by ionizing radiation. That DSBs are located non-randomly along chromosomes has been confirmed by recent pulse field gel electrophoresis data for size distributions of DNA fragments after high doses of high LET radiation, with clustering apparently occurring even at extremely large scales, up to several Mbp.

We therefore extended the standard random-breakage model for DNA fragment-size distributions to a more general "clustered-breakage" formalism, which can take into account correlations of DSB locations along a chromosome.

The new formalism is based on a single-track probability distribution, describing the DNA fragment-size pattern due to a single primary high-energy particle, a pattern determined by track structure and chromatin geometry. Multi-track fragment-size distributions are derived mathematically from the single-track distribution, so that dose-response relations are obtained.

The clustered-breakage formalism is applicable to any chromosomal geometry and any radiation track structure. It allows extrapolations of high-dose data to the much lower doses of interest for most applications. Interestingly, the dose-response relations for fragment size become non-linear when clusters from different tracks interlace, as can occur for high doses and large sizes.

When applied to recently published data for irradiation of mammalian cells with ions of LET around 100 keV/ μm , it implies a pattern of megabase-scale DSB clusters, each containing a number of DSBs and corresponding to an extremely large multiply damaged chromatin site. Importantly, estimates of DSB yield are markedly increased by resolving such clusters into individual DSBs, so that earlier estimates that the RBE for DSB induction by alpha particles is around 1 are probably underestimates.

The dose-response relations for fragment size become non-linear when clusters from different tracks interlace, as can occur for high doses and large sizes.

ATM INACTIVATION RESULTS IN ABERRANT TELOMERE CLUSTERING DURING MEIOTIC PROPHASE

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Ataxia telangiectasia (A-T) is an autosomal recessive disorder characterized by progressive neurological degeneration, premature aging, growth retardation, specific immunodeficiencies, telangiectasia, high sensitivity to ionizing radiation, genomic instability and cancer progression. Ataxia telangiectasia individuals frequently display gonadal atrophy and *Atm*^{-/-} mice show spermatogenic failure due to arrest at prophase of meiosis I. Cells derived from A-T patients exhibit a variety of abnormalities in culture such as cytoskeletal defect, hypersensitivity to ionizing radiation and higher requirement for serum growth factors. They also show a prominent chromatin defect at chromosome ends in the form of chromosome end-to-end associations (also known as telomeric associations, TA) seen at metaphase. Chromosome end associations correlate with genomic instability and carcinogenicity, and involve telomeres. Telomeres consist of repetitive DNA and proteins which protect chromosome ends from exonucleolytic attack, fusion, and incomplete replication. Telomere erosion in a variety of cancers and cell lines has been found to lead to chromosome end associations that could contribute to genomic instability and gene amplification.

It has been suggested that mammalian terminal (TTAGGG)_n repeat arrays interact with the nuclear matrix. Whether ATM gene effectors influence the interaction of telomeres with the nuclear matrix is not yet known. The ATM gene shares the PI-3 kinase signature of a growing family of proteins involved in the control of cell cycle progression, processing of DNA damage and maintenance of genomic stability. The protein shows similarity to several yeast and mammalian proteins involved in meiotic recombination and cell cycle progression, e.g., the products of MEC1 in budding yeast and rad3+ in fission yeast, and the TOR proteins of yeast and mammals. In yeast, non-telomeric DNA created by enzymatic cleavage leads to genomic instability and cell cycle arrest. Because of ATM homology to TEL1 and rad3+ mutants of yeast, it has been suggested that mutations in ATM could lead to defective telomere maintenance. We have recently reported an alteration in both basal and radiation-induced telomeric associations, and in mean telomere length in isogenic ATM cells demonstrating a direct link between ATM function and telomere maintenance. A possible hypothesis explaining the defective telomere maintenance in A-T cells could be due to altered interactions between the telomeres and the nuclear matrix. An altered interaction between telomeres and the nuclear matrix, and nucleosomal periodicity in telomeric chromatin was found in somatic cells derived from A-T individuals.

Telomeres have also been considered as key structures of meiotic chromosomes. Meiosis is a specialized cell division that ensures the proper segregation of genetic material and formation of viable haploid gametes. The most critical events of meiosis occur during prophase I, when homologous chromosomes get aligned (prealign), synapse (pair) and recombine with each

other. During early meiotic prophase telomeres redistribute and accumulate at a limited sector of the nuclear membrane to form a chromosomal bouquet. A number of studies suggest that bouquet formation mediates prealignment of homologues and thereby facilitates synapsis. The only known telomeric proteins that have been implicated in bouquet formation are the products of *Taz1* of fission yeast and *Ndj1/Tam1* of budding yeast. Telomeric and chromosomal movements take place during meiotic prophase, with telomeres congregating to form a tight cluster during the leptotene/zygotene transition. Since the ATM protein has been implicated in telomere metabolism of somatic cells, we have set out to investigate the effects of *Atm* inactivation on meiotic telomere behavior.

ATM is a multifaceted protein and is part of a signaling pathway that responds to DNA damage. This pathway involves p53 as well as c-Abl as A-T null cells are defective in activation of p53 and c-Abl. Abrogation of ATM function leads to telomere associations at metaphase and to disruption of spermatogenesis due to a meiotic prophase arrest. Immunolocalization studies have indicated that the ATM protein is associated with sites along the synaptonemal complex, which are thought to be involved in meiotic recombination. Since telomere clustering at meiosis is thought to bring about prealignment and pairing of homologues (see above) and the *Atm* mutation influences mitotic telomere behavior, we investigated meiotic telomere distribution in spermatocytes of mice disrupted in *Atm* function. FISH and synaptonemal complex (SC) immunostaining of structurally preserved spermatocytes revealed that telomere clustering occurs aberrantly in *Atm*^{-/-} mice. Spermatocytes of *Atm*^{-/-} mice frequently displayed locally accumulated telomeres with stretches of SC near the clustered chromosome ends. This contrasted with telomere distribution in spermatocytes of normal mice, where telomeres were clustered at leptotene/zygotene, while pachytene nuclei had telomeres dispersed over the nuclear envelope. When we examined telomere/nuclear matrix interactions in spermatocytes I, a significant difference was observed in the ratio of soluble versus matrix associated telomeric DNA sequences between meiocytes of *Atm*^{-/-} and control mice. Furthermore, we determined the telomerase activity, expression of TRF1 and several other genes in spermatocytes I of *atm* null and control mice. Although *Atm*^{-/-} mice have telomeres aberrantly associated with nuclear matrix, the expression of TRF1, 588 other known genes, and telomerase activity were identical among *Atm*^{-/-} and control mice. Our results suggest telomere clustering and its failure of resolution in *Atm* null mice links with aberrant synapsis and meiotic arrest. We propose that ATM inactivation influences interactions of telomeres with the nuclear matrix and thereby meiotic telomere clustering.

ATM FUNCTION INFLUENCE CHROMOSOME END-TO-END ASSOCIATIONS AND TELOMERE LENGTH, BUT NOT TELOMERASE ACTIVITY

Lubomir B. Smilenov and Tej K. Pandita

Ataxia telangiectasia (AT) is a rare human autosomal recessive disorder with a wide variety of phenotypic manifestations. AT patients exhibit progressive cerebral ataxia with degeneration of Purkinje cells, hypersensitivity to ionizing radiation and radiomimetic drugs, thymic hypoplasia, growth retardation, premature aging and gonadal atrophy.

The ATM gene product, which is defective in ataxia telangiectasia has been implicated in mitogenic signal transduction, chromosome condensation, meiotic recombination and cell cycle control. Because of ATM gene homology to the TEL1 gene in yeast, mutations of which lead to shortened telomeres, it has been suggested that mutations in ATM gene could lead to defective telomere maintenance. To test the hypothesis that the ATM gene product is involved in telomere metabolism, we examined telomeric associations (TA), telomere length, and telomerase activity in RKO cells expressing dominant-negative fragments of the ATM gene. To determine whether ATM function influences TA, we examined metaphase spreads and found that all of the RKO cells expressing the dominant-negative fragments exhibited a three- to six-fold higher frequency of cells with TA compared to parental and control RKO cells. This abnormal telomeric association phenotype was also manifested in G1- and G2-phase cells as determined by using the premature chromosome condensation technique. Since gamma irradiation triggers TA in A-T cells we also evaluated TA in these cells after radiation treatment and found that the frequency of cells with TA also increased in the RKO cells expressing the dominant-negative ATM fragments compared to control cells.

Telomeric association frequencies were also evaluated in A-T cell line expressing the complementing kinase domain fragment. The kinase domain fragment complemented A-T cells for various cellular functions and had two to four fold lower frequencies of cells with TA than the parental or control-transfected cells in metaphase as well as G1 and G2. Similarly, radiation-induced TA were also significantly lowered by expression of the kinase fragment in AT cells. The correlation between manipulated ATM function and basal and irradiation-induced TA, demonstrated that ATM function influences telomere metabolism.

About seventy five RKO clones expressing the dominant-negative fragment had significantly shorter mean telomere length. Further, when mean telomere length was evaluated with increased population doublings in the RKO cells expressing the dominant-negative fragments, both a decrease in telomere signal and a shortening of telomere length (loss of about 22 bases per doubling) was observed with the increase in the population doublings. The telomere length did not change over 136 population doublings in the control RKO cells. These results suggest that ATM function influences telomeric length.

Since manipulations of ATM function appeared to influence TA as well as telomere length, we determined whether telomerase activity is influenced by ATM gene product. Telomerase activity was measured by TRAPeze (Oncor) and TelomerasePCR-ELISA (Boehringer Mannheim) protocols. A linear increase in the telomerase activity was found with the

increase in the protein concentrations with no differences in telomerase activity per unit of protein among the RKO cells with and without fragment of ATM gene. Similarly, no differences in telomerase activity were seen among GM 5849 cells with or without ATM complementing activity.

Chromosome end associations may not be dependent upon the telomere size, because higher frequency of cells with chromosome end associations were seen in all clones of manipulated RKO cells, with short as well as long telomeres. It has been suggested that a complex of specialized proteins that bind telomeric DNA conceal the termini of natural chromosome ends. Thus, telomeres hide natural chromosome ends from factors that act on DNA and are likely to be resistant to exonucleases and ligases. Telomere binding factors have been characterized in yeast and in several ciliates and have also been reported in vertebrate cell extracts. It is possible that the function of telomere binding factors may be influenced in their function by the ATM-related gene products

Recently three telomere binding proteins namely, TRF1, TRF2 and PIN2 have been identified. They have DNA binding properties with TTAGGG repeats in vitro irrespective of the presence of a DNA terminus, properties which are consistent with its presence along the ends of chromosomes. Human TRF1 has been implicated in the regulation of telomere length. We determined the status of human TRF1 in RKO cells with and without fragments of ATM and found TRF1 RNA was expressed in all of the clones independent of ATM functional status and no mutations were detected by cold single strand conformational polymorphism analysis. The possibility remains, however, that ATM influences hTRF1 function.

Regulation of telomere length has been a focus of research in carcinogenesis. It is generally believed that changes in telomere dynamics play a role in malignant transformation of human cells in vitro. Primary fibroblasts from humans and mice with defective ATM genes grow poorly in culture and appear to undergo premature senescence in culture. In addition, AT patients exhibit clinical signs consistent with a premature aging phenotype. Finally, ATM has significant regions of homology with the yeast TEL1 gene, which is involved in maintaining telomere length. All these observations suggest a potential influence of ATM gene on telomere metabolism. Our observations in both basal and radiation-induced telomeric associations and in mean telomere length in isogenic cells with manipulated ATM function demonstrate a direct link between ATM function and telomere maintenance.

ALTERED TELOMERE NUCLEAR MATRIX INTERACTIONS AND NUCLEOSOMAL PERIODICITY IN CELLS DERIVED FROM ATAXIA TELANGIECTASIA PATIENTS BEFORE AND AFTER IONIZING RADIATION TREATMENT

Tej K. Pandita, Satin G. Sawant and Lubomir B. Smilenov

Ataxia telangiectasia (A-T) is a rare autosomal human recessive disorder characterized by progressive neurological degeneration, growth retardation, premature aging, telangiectasia, specific immunodeficiencies, high sensitivity to ionizing radiation, gonadal atrophy, genomic instability and cancer predisposition. Cells derived from A-T individuals exhibit a variety of abnormalities in culture such as a higher requirement for serum factors, hypersensitivity to ionizing radiation and cytoskeletal defects. They also show a prominent chromatin defect at chromosome ends in the form of chromosome end-to-end associations seen at different phases of the cell cycle. Chromosome end associations involve telomeres and these are composed of repetitive DNA sequences of TTAGGG arrays concealed by a complex of specialized proteins that protect ends from exonucleolytic attack, fusion and incomplete replication. Telomeric associations correlate with genomic instability and carcinogenicity.

There is growing evidence suggesting that both the shielding of telomeric ends and their elongation by telomerase are dependent upon telomere binding proteins. Mammalian telomeres are packaged in telomere specific chromatin. Human and mouse cell lines have their telomeric tracts attached to the nuclear matrix, which is a proteinaceous subnuclear fraction. There is a difference in nucleosomal organization of telomeres as compared to bulk DNA and telomeric histone H4 is hypoacetylated. Telomere length homeostasis in yeast requires the binding of a protein along the telomeric tract and changes in the telomeric matrix binding site occurs at least once in every kb of the telomeric tract in tumor derived cell lines. It has been suggested that mammalian telomeres have frequent multiple interactions with the nuclear matrix over a large domain that encompasses the entire telomeres of most of the chromosome ends. Whether the ATM gene influences the interaction of telomeres with the nuclear matrix is not yet known.

Telomere nuclear matrix interactions and nucleosomal periodicity in A-T and normal individuals were carried out on primary fibroblasts. The telomeres of these cells shorten during proliferation in culture. It is possible that the interactions of telomeres with the nuclear matrix may depend upon the length of the terminal restriction fragment (telomere length) of the chromosomes. Therefore, we determined the mean telomere length of each cell type at the time their nuclear matrix interaction and nucleosomal periodicity were examined. We found the mean telomere length of A-T primary cells (GM5823, GM2052) were comparable to those of the controls (AG1522, C21F).

Telomere-nuclear matrix association

Telomeres are attached to the nuclear matrix. To characterize the nature of telomere anchorage in different cell types, plateau phase cells were processed according to the LIS procedure. A-T cells (GM5823, GM2052) have more than 95% of the telomeric DNA attached to the nuclear matrix where as in control cells (AG1522, C21) about 60% is

attached. The ratio between the fractions of soluble versus nuclear matrix attached telomeric DNA is about 1:19 in A-T cells as compared to 1:1.5 in normal cells. These results suggest that the major portion of telomeres in A-T cells are associated with the nuclear matrix.

Influence of ionizing radiation on telomere-nuclear matrix associations

To determine whether ionizing radiation treatment causes release of nuclear matrix bound telomeric DNA, plateau phase cells were treated with a biologically relevant dose of 5 Gy of ionizing radiation and analyzed for the ratio of soluble versus matrix attached telomeric DNA. No change in the ratio of soluble versus attached fractions of telomeric DNA was seen immediately after treatment with ionizing radiation, neither in the control and nor in the A-T cells. However, a significant increase in telomeric DNA within the soluble fraction was seen in A-T cells after one hour post treatment, whereas no such change in the release of matrix attached telomeric DNA was found in normal cells. An increase in the soluble fraction of the telomeric DNA in normal cells was seen only after one hour of treatment with doses above 10 Gy of ionizing radiation.

Nucleosomal organization in telomeric chromatin

To determine the nucleosomal organization of the telomeric arrays of TTAGGG, we digested the nuclei with different concentrations of MNase and detected telomere repeats by Southern analysis using the TTAGGG probe. We determined the organization of the TTAGGG repeat sequences by comparing the nucleosomal periodicity of the bulk chromatin with the TTAGGG. We found relatively higher MNase digestion of chromatin in A-T cells as compared to normal cells. This suggests that nucleosomes in A-T cells are spaced loosely, thus leading to an altered periodicity. Since the ethidium bromide staining represents bulk chromatin, we were interested in examining nucleosomal patterns in telomere regions. Normal cells have a telomere ladder, each containing partials up to seven subunits. A different result is seen in A-T cells where the telomeric pattern revealed a less extensive MNase periodicity and telomeric nucleosomal arrays with up to three subunits were detected. These results suggest that the telomeric nucleosome arrays in A-T cells might be less uniformly spaced or might extend over a smaller region than the arrays.

To determine how the altered nucleosomal periodicity in telomeres seen in A-T cells respond to ionizing radiations, we examined the influence of radiation treatment on nucleosomal compaction in telomeres. There was no change in the nucleosomal size of bulk chromatin as revealed by ethidium bromide staining. However we observed the disappearance of the mononucleosome band in the telomere region of A-T cells one hour after gamma ray treatment, whereas no such change was seen in normal cells. The disappearance of mononucleosome band in A-T cells suggests that ATM influences the response of telomere chromatin to ionizing radiation.

Telomere Repeat Binding Factors

To determine whether the abnormalities in telomere nuclear matrix interactions and nucleosomal periodicity seen in A-T cells are correlated with alterations in telomere binding factors, we first analyzed the expression of telomere repeat binding factors (TRF1 and TRF2) in A-T fibroblasts. We used the RT-PCR approach to determine the expression of TRF1 and TRF2. We found comparable levels of expression of TRF1 and TRF2 in A-T and normal control cells. Although the expression of TRF1 and TRF2 were identical between A-T and

control cells, it is possible that mutations in these genes could lead to altered interactions of telomeres with the nuclear matrix. Therefore, we carried mutational analysis of TRF1 and TRF2 genes in A-T and control cells. Analysis of TRF1 and TRF2 cDNA in A-T cells by cold SSCP protocol revealed no mutations.

Since the expression of telomeric binding proteins in A-T cells was comparable to normal cells, and no mutations were found in TRF1 and TRF2 genes, we were then interested to determine whether TRF1 and TRF2 proteins are localized in the nucleus. We performed the immunostaining and found that both TRF1 and TRF2 proteins are localized in the nucleus in both A-T and control cells. These observations suggest that alterations in TRF1 and TRF2 are not the cause for altered interactions of the telomeres with the nuclear matrix and nucleosomal periodicity changes in A-T cells.

In an attempt to identify the gene products that might be involved with the altered interactions of telomeres with the nuclear matrix in A-T cells, we used the ATLAS cDNA microarray to analyze the expression of genes. The Atlas Human cDNA expression array contains 588 genes of different classes, including cell cycle control regulators, tumor suppressors, oncogenes, ion channel and transport proteins, intracellular signal transduction modulators and effectors, apoptosis-associated proteins, stress response proteins, DNA synthesis/repair /recombination proteins, transcriptional factors, DNA binding proteins, cell receptors, cell surface antigens/adhesion, extracellular cell signaling proteins and housekeeping genes. cDNA expression array membranes were hybridized to the cDNA made from A-T and control cells. The expression profiles of primary fibroblasts of A-T and normal control were compared using different amounts of polyA⁺ RNA that were used to make ³²P labeled cDNA and subsequently hybridized separately to array membranes. No significant differences in the expression of 588 genes on the array were found between A-T and normal control cells.

What are factors that might influence chromosome end association? One possible factor is loss or shortening of telomeres that could lead to chromosome end to end associations as suggested by Counter et al., (1992). Another possible factor is altered chromatin structure. In our recent studies, we found that the frequency of cells with chromosome end associations is higher in G1-phase than in G2-phase followed by metaphase, and for each phase of the cell cycle, the frequency of cells with end associations was significantly higher in A-T than in normal cells (Pandita et al., 1995). It is probable that the end associations seen at mitosis reflect a continuation of interphase chromosome behavior, indicating perhaps interactions or linkages between chromosome ends and the nuclear matrix. Since the telomeric signals are seen at the chromosome end associations sites (Pandita et al., 1995), it is possible that the chromosome end associations are the consequences of the failure of the nuclear matrix withholding the telomeres together. The telomeric signals at the chromosome end association sites in A-T cells suggest that chromosome end associations could be the primary event that subsequently leads to the loss of telomeres. This interpretation is consistent with the recent findings of van Steensel et al., (1998) who also reported that the telomeric signals were present at sites of chromosome end associations and shortening of telomeres is not prerequisite for chromosome end associations. Telomeric signals at the chromosome end association sites and changes in the frequency of cells with chromosome end associations

through the cell cycle raise the possibility that A-T cells might have an altered nuclear matrix, leading to defective interactions between telomeric DNA and the nuclear matrix.

Telomeres are important components of chromosomes as they have been implicated in several cellular functions involved in aging and cancer development. Telomeres have been shown cytologically as well as biochemically to be tethered to the nuclear matrix. The nuclear matrix is nonchromatin scaffolding of the interphase nucleus and is isolated by removing most of the nuclear DNA and RNA, along with histones and loosely bound proteins (Berezney et al., 1995). Our present study shows that telomeres of primary fibroblasts are associated with the nuclear matrix and such observations are consistent with the previous observations of Luderus et al., (1996). However, we found a significant difference in the ratio of the bound versus soluble fraction of telomeric DNA between A-T and normal control cells. This difference could be attributed to differences in the interactions between telomeric DNA and the nuclear matrix. One reason for altered interactions of telomeric DNA binding to the nuclear matrix could be modified nuclear matrix structure. The other possibility is a difference in telomere length. Our studies support the idea that the nuclear matrix composition could be responsible for differences seen in telomere anchorage, but not telomere length as the A-T and normal cells examined had telomeres of similar size. The altered anchorage of telomeric DNA to the nuclear matrix in A-T cells influences the release of matrix bound telomeric DNA after treatment with ionizing radiation. In contrast to normal cells, the soluble fraction of the telomeric DNA changed dramatically in A-T cells after treatment with gamma rays. Release of telomeric DNA from the nuclear matrix strengthens the idea that A-T cells have a defective nuclear matrix composition.

Genomic DNA is compacted within the nucleus as chromatin (nucleoprotein complex) and the basic unit of chromatin is the nucleosome that consists of 146 bp of DNA wrapped around an octamer of histones (Kornberg and Lorch, 1995). The nucleosomal size in bulk as well as telomeric DNA is similar among A-T and normal cells. The differences lie in the periodicity of the nucleosomes in the telomeric region in A-T versus normal cells. This is further indicative of altered nuclear matrix composition. Nucleosomes in telomeres of A-T cells are loosely spaced, and this state of nucleosomal periodicity in telomeres could not be attributed to the length of telomeres, as the telomeres of the A-T and normal cells examined were of similar size.

The differences in A-T and normal cells for radiation response could partly be attributed to altered chromatin organization as is evident from the differences in nucleosomal periodicity and nucleosomal compaction studies after ionizing radiation treatment. When nucleosomal compaction is examined in A-T and normal fibroblasts after treatment with ionizing radiation, bulk chromatin does not show any distinct alterations; however nucleosomal compaction was influenced only in the telomeric region of A-T cells, but not in normal fibroblasts. Why are alterations in the compaction of the nucleosomes seen in telomeric DNA and not in the bulk chromatin? The reason for this could be that the major portions of telomeric DNA are attached to the nuclear matrix, whereas only a fraction of the bulk DNA is associated with it. As discussed above, the telomere/nuclear matrix interactions were different in A-T cells compared to normal cells. Therefore, it is possible that an altered nuclear matrix could influence specifically the matrix-associated nucleosomes. This could be the reason why alterations in the compaction of the nucleosomes were detected in nucleosomes of telomeres of A-T cells only. Therefore, our studies support the idea that the

altered chromatin responds to DNA damage in a different way and thus influences the nucleosomal compaction only in A-T cells.

At present it is not clear how differences in the interaction of telomeric DNA with the nuclear matrix, and nucleosomal periodicity observed in A-T and normal cells could be due to differences in telomere binding factors. Only two human telomere binding factors (hTRF1 and hTRF2) are known and both have myb domains. When these genes were analyzed in A-T and normal cells for their expression, mutations and localization of their protein product, no differences were found. Furthermore, when expression of the 588 genes of the cDNA ATLAS array of Clonotech among A-T and normal fibroblasts were compared, no differences were noticed. These observations suggest that there are no specific changes in the expression of most genes. Therefore, the differential response to ionizing radiation treatment could not be attributed to specific alterations in the expression of these genes. Information about the interactions of the ATM gene with others is limited. However, it has been shown that ATM interacts with c-Abl, p53 and beta-adaptin. It remains to be established how such gene could influence the nuclear matrix and then the function of the gene.

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Figure 1. Nucleosomal periodicity of normal (AG1522), and A-T (GM5823) cell lines. Nuclei of each cell line were prepared as described recently (Smilenov et al., 1998). Digestion of chromatin DNA with micrococcal nuclease leads to the generation of a ladder of DNA fragments as resolved on 1.5% agarose gel. The MNase concentrations used were 0 (lane 1), 125 (lane 2), 250 (lane 3), 500 (lane 4), 1000 (lane 5), 2000 (lane 6), 4000 (lane 7) and 8000 (lane 8) U/ml. The position of the mononucleosomal band is indicated at the left of each ethidium bromide figure. Figures 4a and 4c represent normal (AG1522) and A-T (GM5823) cell lines. MNase digestions are compared between A-T and normal cell lines. A-T cell lines show more susceptibility to MNase digestion than normal cells as is evident when lanes 2 and 3 of each figure are compared. Figures 4b and 4d are the autoradiographs visualize telomeric DNA as detected by TTAGGG probe. Two kinds of chromatin are seen. Figure 4b represent normal cell line that has telomeres with about 7 nucleosomal subunit and Figure 4d represents AT cell line that has telomeres with about 3 nucleosomal subunits. The position of the mononucleosomal bands is indicated to the right of the autoradiograph.

MOLECULAR CLONING AND CHROMOSOMAL LOCALIZATION OF CHINESE HAMSTER TELOMERIC PROTEIN CHTRF1. ITS POTENTIAL ROLE IN CHROMOSOMAL INSTABILITY

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Telomeres, the protein-DNA complexes at the ends of chromosomes, are essential for chromosome stability in eukaryotic cells. Telomeres cap and protect the termini of chromosomes from degradation and end-to-end fusions. Mammalian telomeres contain long arrays of TTAGGG repeats that are bound with specialized protein complexes and have several functions. Telomere specific TTAGGG repeat sequence can be maintained by the telomere specific RNP polymerase, telomerase. Three telomere binding proteins namely, TRF1, TRF2 and PIN2 have been identified. They have DNA binding properties with TTAGGG repeats in vitro irrespective of presence of a DNA terminus, properties which are consistent with its presence along the ends of chromosomes. TRF1 has been implicated in the regulation of telomere length and TRF2 has been characterized as a distant homologue of TRF1, with similar binding domain structure and TTAGGG binding activity. Interestingly, the comparison between the mouse and human TRF1 show lower than expected identity, supporting the idea that mammalian telomeric proteins evolve rapidly (Broccoli et al., 1997). The Chinese hamster and mouse are closely related species and the characterization of Chinese hamster TRF1 could confirm this notion.

Chinese hamster cell lines have been widely used to explore the mechanism of gene expression and amplification. They frequently have altered karyotypes, often with no homologous pairs of chromosomes and a high frequency of cells with telomere fusions. The Chinese hamster is also an interesting model for telomere studies as its chromosomes contain large blocks of interstitial as well as terminal TTAGGG repeats. It has been reported that interstitial telomere like sequences are preferentially involved in chromosome breakage and exchange in rodent cells. The frequency of ionizing radiation induced chromosome aberrations have been reported to be higher in the region of interstitial telomeric repeats. These regions have also been shown to have very high rates of recombination. Our studies revealed that interstitial bands of TTAGGG arrays undergo spontaneous amplification (Pandita and DeRubeis, 1995). The reason for this genomic instability and whether it is regulated by TRF1 is not known. A possible interaction between the TRF1 and the TTAGGG repeats may provide more information on the role of the telomeric binding factors.

We cloned the Chinese hamster TRF1 from the cDNA of the Chinese hamster ovary cells and the Chinese embryonic cells by using human TRF1 specific primers. The single 1.3 kb PCR product was subcloned in pGEM-T vector. Conceptual translation of the chTRF1 open reading frame and the alignment of this sequence with the human TRF1 and mouse TRF1

open reading frames are shown in the Figure 1. The sequence analysis of chTRF1 revealed that the gene encodes an open reading frame of 438 amino acid protein. The human and Chinese hamster TRF1 proteins are very closely related as they show 97.5% overall amino acid identity. This is in contrast to mouse TRF1 which shows a considerable sequence divergence.

The Chinese hamster TRF1 has a N-terminal acidic domain, a middle TRF-specific domain and Myb HTH domain at COOH-terminal, similar to that of human TRF1 and mouse TRF1. An absolute sequence identity between human and the Chinese hamster TRF1 was seen in the Myb domain, with the TRF-specific domain and the acidic domain showing 98% and 88% identity respectively. Chinese hamster TRF1 has a putative nuclear localization signal in between the TRF binding sites and the Myb-domain similar to the human TRF1. Further, amino acid sequence comparison also revealed that there are several putative phosphorylation sites that are conserved between the Chinese hamster, mouse and human TRF1 proteins. The greater similarity between the human TRF1 and Chinese hamster TRF1 than compared to mouse TRF1 and Chinese hamster TRF1 was unexpected. The amino acid identity between the mouse and Chinese hamster TRF1 is 83% in the TRF specific domain, 82% in the Myb domain and about 40% in other regions.

To determine the subchromosomal localization of the Chinese hamster TRF1, we performed fluorescent in situ hybridization of a biotin labeled Chinese hamster TRF1 probe to Chinese hamster metaphase and localized the gene on chromosome 5.

Since conceptual translation of the open reading frame of TRF1 of Chinese hamster and human showed 97.5% amino acid identity, we were interested to determine whether Chinese hamster TRF1 produces a protein product similar to human TRF1, therefore, we carried out an in vitro translation. The cloned Chinese hamster TRF1 cDNA was capable of directing the expression of an in vitro translation product which was indistinguishable from a human TRF1 cDNA product.

TRF1 binds efficiently to arrays of duplex TTAGGG repeats, irrespective of the presence of a DNA terminus. Since Chinese hamster cells have blocks of TTAGGG repetitive bands, we wished to determine whether Chinese hamster TRF1 is associated with these bands and could CHO cells be used as a model for understanding the interactions between TRF1 and TTAGGG repeats. We applied FISH using biotinylated TTAGGG probe to detect the TTAGGG arrays and found that most of the cells have about 18 to 21 readily cytologically detectable arrays of telomeric bands of TTAGGG repeats on metaphase and anaphase chromosomes. Further, we determined the localization of the TRF1 protein in CHO cells by using several antibodies against TRF1. We did not see immunostaining of TRF1 antibodies at the interstitial blocks of TTAGGG arrays. Immunofluorescent signals of TRF1 antibodies were seen only at a few metaphase chromosome ends of Chinese hamster cells. These observations suggest that TRF1 protein does not bind the interstitial telomeric bands of Chinese hamster cells. One possible reason could be that telomeric bands in CHO cells may have a different structure compared to TTAGGG arrays reported in human and mouse.

To determine the nucleosomal organization of telomeric arrays of TTAGGG, we digested the nuclei with different concentrations of MNase and detected telomere repeats by Southern analysis using the TTAGGG probe. Telomeric bands yielded nucleosomal ladders that are similar to the bulk chromatin products, each containing partial upto eight units. The size of

the nucleosome from the interstitial TTAGGG arrays is about 146 bp which is similar to bulk DNA. These observations suggest that TTAGGG sequences are organized in nucleosomal pattern similar to bulk DNA. This could provide a possible explanation for why TRF1 does not bind to the interstitial block of TTAGGG. These observations support the idea that TRF1 may have a special role to play at the chromosome ends, the telomeres.

The evolutionary reason for divergence of mouse TRF1 from that of Chinese hamster and human TRF1 gene sequences is not clear at present. A possible reason is that the mouse telomeres are much longer than the telomeres of human and Chinese hamster and thus the organization of mouse telomeres may be different from the relative short ones of human and Chinese hamster. It is possible that Chinese hamster TRF1 and human TRF1 interact with similar proteins that are then bound to relatively short telomeres as compared to the mouse telomeres which interact with other types of proteins bound to longer telomeres. What are the factor/s that influence the binding of TRF1 with TTAGGG in CHO cells needs to be determined.

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CORRELATION BETWEEN TELOMERASE ACTIVITY AND TELOMERE LENGTH IN HUMAN BREAST EPITHELIAL CELL LINES

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Normal somatic cells have a highly restricted cell proliferative ability and senesce after a certain number of cell divisions, whereas neoplastic cells undergo deregulated proliferation. It has been estimated that cancer evolves through the combined effects of 5 or 6 independent mutations (Loeb, *Cancer Research* 54: 5059, 1994). Telomerase is a ribonucleoprotein DNA polymerase that plays a key role in telomere synthesis by adding telomere repeats onto chromosome ends to compensate for sequence loss during DNA, is involved in tumorigenesis. The G+T rich sequence of TTAGGG repeats vary in human telomeres resulting in telomere length heterogeneity (Blackburn, *Nature* 350: 569, 1991). It is generally believed that changes in telomere dynamics plays a role in malignant transformation of human cells *in vitro*.

The aim of this work was to determine the correlation between telomerase activity and telomere length in transformed and tumorigenic human breast epithelial cells (HBEC) induced by Benz(a)pyrene (BP) and ionizing radiation. We examined the immortalized MCF-10F cell line (Soule et al. *Cancer Res.* 50: 6075, 1990) and its derivatives treated with BP (Calaf and Russo, *Carcinogenesis* 14: 483, 1993) and/or transfected with the c-Ha-ras oncogene (Calaf et al., *Intem. J. Oncology* 6: 5, 1995) followed by a single 3Gy dose of γ irradiation.

Telomerase activity was analyzed by the Telomeric Repeat Amplification Protocol (TRAP), a photometric enzyme immunoassay for the detection of telomerase activity (Kim et al., *Science* 266: 201 1, 1994). The absorbance of the samples was measured at 450 nm using an ELISA microplate reader. In order to measure telomere lengths, the terminal restriction fragments (TRF) were measured by extraction of DNA from control and tumorigenic cells (Pandita et al., *Oncogene* 13: 1423, 1996). The DNA was then digested with the restriction enzymes, RsaI and HinfI, which do not cut TTAGGG sequences, and was fractionated on a 8% agarose gel. The membranes were then hybridized with 3'P-end labeled TTAGGG and the mean TRF length was determined by densitometric analysis.

Although the transformed and tumorigenic breast cells examined in this study are all derivative of the immortalized, parental MCF-10F cells, they show difference in their expression of both the telomeric length and telomerase activity. The transformed MCF-10F-c-Ha-ras and MCF10F-c-Ha-ras-3Gy showed similar telomere lengths and telomerase activities as the parental MCF cells and HeLa cells. In contrast, the BP-tumorigenic variants examined all demonstrated shorter telomeres (Figure 1 and 2) and lower telomerase activities (Figure 3) than the controls and the c-Ha-ras transfected cells.

While telomeres, in general, do shorten during tumorigenic conversion as tumors with shorter telomeres than control tissues have been identified in many types of cancers, there are exceptions, e.g. certain leukemias and lung cancer have very long telomeres. In contrast, telomerase activity is readily detected in over 90% of cancer tissues examined (Kim et al.

Science 266: 2011, 1994). Previous studies from this laboratory have demonstrated that telomerase activity correlates with metastatic potential in tumorigenic human bronchial epithelial cells transformed by alpha particles (Pandita et al., *Oncogene* 13: 1423, 1996). In contrast, no correlation was found between telomere length and the different transformation stages identified in bronchial carcinogenesis. Our present findings of a reduced telomerase activity among tumorigenic MCF- 10F cells transformed by the chemical carcinogen, BP, although surprising, is not unique since malignancies that are telomerase negative have also been reported in retinoblastomas and chronic lymphocytic leukemia. The low telomerase activity in our tumor samples might either reflect a clonal selection of parental MCF cells with low telomerase or that the maintenance of critical telomere length in these cells is mediated by a telomerase independent pathway. Further studies to elucidate these possible mechanisms are currently underway.