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Restoration of Senescence in Breast and Ovarian Cancer Cells Following the Transfer of the YAC Carrying SEN6A Gene Located at 6q16.3

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Abstract. *We previously located a senescence gene locus (SEN6A), at chromosome 6q14-21 by a functional strategy using chromosome transfer into immortal ovarian tumor cells. To further elucidate the SEN6A locus, intact chromosome 6 or 6q was transferred into rat ovarian tumor cells and a panel of immortal revertant clones of senescent cells was generated. The panel of independent colonies as well as mixed populations of revertant cells was analyzed for the presence or absence of chromosome 6 specific markers. These investigations led to the identification of a fine deletion of approximately 1cM at chromosomal interval 6q16.3. A contiguous stretch containing five yeast artificial chromosome (YAC) clones was constructed across the deleted region. The non-chimeric YAC clones were retrofitted and transferred into mouse A9 cells by spheroplast fusion to generate YAC/A9 hybrids. YAC DNA present in YAC/A9 hybrids was subsequently transferred by microcell fusion into immortal tumor cells, and the hybrid cells were characterized for their senescence phenotype. Using this functional strategy, the transfer of YAC clone 966b10 was shown to restore senescence in both rat and human ovarian and breast tumor cells. Our results demonstrate that the SEN6A gene is carried on a 1 Mb YAC, 966b10, which maps at 6q16.3.*

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Ovarian cancer is the most common gynecological malignancy and fifth leading cause of cancer mortality among women in the USA (1). Approximately 90% of ovarian cancer cases are sporadic, while 10% are genetic in nature. Cytogenetic and loss of heterozygosity (LOH) studies have revealed consistent rearrangements of chromosome 6 in ovarian cancer cells (2-4). The rearrangements of 6q have also been implicated in a variety of other carcinomas such as breast, prostate, salivary gland, melanoma, hematological malignancies and hereditary mixed polyposis syndrome (5). The immortalization of human normal diploid fibroblasts by simian virus 40 (SV40) has also been attributed to rearrangements of chromosome 6q (6, 7).

The chromosomal intervals 6q16-21, 6q22-23 and 6q25-27 have been identified as active targets for sequence rearrangements in a variety of carcinomas (8-10). These regions of 6q are suggested to harbor multiple tumor suppressor genes. However, the individual or synergistic contribution of these three regions toward tumorigenesis has not been adequately evaluated. The evidence for the role of chromosome 6 in the transformation of human and rodent cells is derived from the studies involving the transfer of normal chromosomes into immortal cell lines and reversal of tumorigenic and/or metastatic potential of malignant melanoma (11, 12), uterine endometrial carcinoma (13) and breast carcinoma cells (14).

Cancer cells acquire unlimited capacity to proliferate by escaping senescence, a form of programmed growth arrest that limits the replicative potential of most somatic cells (15, 16). However, tumor cells or SV40 immortalized cells can

gain senescent phenotype following the transfer of normal human chromosome 6 (17-19). Interestingly, some of these senescent cells revert back to an immortal phenotype due to the loss of expression or deletion/inactivation of the senescence gene in the donor chromosome. Identification of minimal deletion in revertants, derived from senescent clones obtained following the transfer of chromosome 6 or 6q into ovarian and breast cancer cells, led to precise mapping of the cell senescence locus SEN6A. We have successfully employed a functional strategy by chromosome transfer for physical mapping of a new senescence locus, SEN6A, in less than 1Mb stretch of DNA at 6q16.3.

Materials and Methods

Cell lines and cell culture. Monochromosomal hybrid cell lines RA6A and RA6q, containing single gpt tagged normal human chromosomes 6 or 6q were used as chromosome donors (unpublished results, RS Athwal; 19). The recipient cell lines for chromosome transfer included rat ovarian tumor cell lines ROSE199 (20) and ROVE 12 (21), rat glioma cell line C6, rat mammary tumor cell line NMU, mouse cell line A9, human breast tumor cell line MCF7 and human ovarian carcinoma cell line SKOV3. Mouse A9 cells were used for the transfer of yeast artificial chromosome (YAC) DNA. The cells were routinely cultured in DF12 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 10 U/ml penicillin and 10 U/ml streptomycin at 37˚C in a 7.5% CO_2/air atmosphere. The microcell hybrids and YAC transfer clones were selected in the presence of 25 μg/ml mycophenolic acid and 70 μg/ml xanthine (MX medium) or in a medium containing 400 μg/ml G418, respectively.

Retrofitting of YAC clones to incorporate neo selectable marker. The YAC clones (Research Genetics, Huntsville, AL, USA) were propagated in 3 ml culture medium containing yeast nitrogen base without amino acids or $(NH4)_2SO_4$ (1.7 g/L); acid-hydrolyzed casein (10 g/L) and adenine hemisulfate (20 mg/L) as previously described (22). The plasmid vector pRAN4, carrying a selectable neo marker, was integrated into YAC966b10 by homologous recombination (23, 24). YAC containing yeast cells were transformed with 1 μg of pRAN4 DNA that had been linearized with BamHI restriction enzyme, and the cells with the retrofitted YAC were selected by growing in adenine deficient medium (25). Each retrofitted YAC clone was verified for the desired genotype (ura-, ade+, trp+) by growing in a medium containing specific combinations of uracil, adenine and tryptophan. Derivative retrofitted clones were analyzed by Southern blot hybridization following pulsed-field gel electrophoresis (PFGE). The presence of neo marker in YAC clones was confirmed by PCR. Four YAC clones were retrofitted with a neo marker for selection in the mammalian host cells, and at least three retrofitted clones were preserved for each YAC.

Transfer of YAC DNA into mammalian cells. YAC DNA was introduced into mouse A9 cells either by spheroplast fusion or by lipofection using LIPOFECTIN reagent (Life Technologies, Carlsbad, CA, USA). For spheroplast fusion, the yeast cells were harvested from 50 ml fresh culture (~3-4×10^7 yeast cells for AB1380 host strain). The cells were washed twice with 1 M sorbitol and suspended in 20 ml SCE medium containing 30 mM β-mercaptoethanol and 1000 U of Zymolyase-20T, and incubated at 30˚C for 35 min or until spheroplast formation. After the incubation, the spheroplasts were centrifuged at low speed (1100 rpm) in a table-top centrifuge at 4˚C for 5 min. The pellet was washed with STC buffer, resuspended in STC buffer and aliquots of cells (1×10^8 spheroplasts) were centrifuged. The pellet was overlaid with 2×10^6 A9 cells, and fusion of cells with spheroplasts was facilitated by the addition of polyethylene glycol (PEG) solution (50% weight/volume, containing 2.5 μM $CaCl_2$, 10% DMSO) for 30 sec at room temperature. The above mixture was then diluted with 5 ml of medium without serum, incubated for 1 h at room temperature, centrifuged (1200 rpm in a table-top centrifuge) for 5 min, re-suspended in medium containing 10% fetal bovine serum and transferred to a 100 mm dish. The medium was replaced after 48 h with selection medium containing 400 μg/ml of G418.

Yeast/YAC DNA was purified using standard procedures and dissolved in TE buffer. For DNA transfection, 1-2×10^5 cells were seeded in 100 mm tissue culture dishes in DF12 medium containing 10% FBS and grown for 24 h. YAC DNA (10-20 μg) was mixed with 300 μl of LIPOFECTIN reagent and added directly to the monolayer of recipient cells in the culture medium. After 48 h of incubation, the contents of each plate of transfected cells was divided into two plates in the selection medium containing 400 μg/ml of G418. The surviving colonies were isolated individually and propagated. Although the transfer of YAC clones by spheroplast fusion was highly efficient with mouse A9, it was unsuccessful with SKOV3, MCF7, ROSE199 and ROVE12 cells. This problem was circumvented by using the following two-step fusion protocol. YAC DNA was first transferred into mouse A9 cells by spheroplast fusion, and the clones were confirmed to have intact YAC DNA by marker analysis. Mouse chromosome carrying intact integrated YAC was further transferred to human (MCF7, SKOV3) and rat (ROSE199, ROVE12) cell lines by microcell fusion (18, 19, 26, 27).

Analysis of revertant clones for mapping the SEN6A locus. DNA from various microcell hybrids and revertant clones was prepared by a standard phenol-chloroform extraction method. Microcell hybrids, all independent revertant clones and mixed cell populations originating from rat cell lines (ROSE199, ROVE12, C6 and NMU) were analyzed for 152 DNA markers (Research Genetics) mapped to 6q13-21. All amplifications were performed in 25 μl reaction volume under the following conditions: initial denaturation at 94˚C followed by 35 cycles of 94˚C (40 sec), 55˚C (30 sec) and 72˚C (2 min). The PCR products were separated by electrophoresis on a 2% agarose gel and photographed after staining with ethidium bromide.

Results

Effect of transfer of chromosome 6q into rat and human tumor cell lines. To confirm the presence and to map cell senescence genes to a defined region of chromosome 6, chromosome 6 and 6q were introduced into a variety of tumor cell lines that included SKOV3, MCF7, ROSE199, ROVE12, NMU and C6. The cells containing chromosome 6 or 6q were enlarged, vacuolated and senesced after a limited number of doublings. These cells displayed extended doubling times varying from 72 h to several days. Figure 1 shows the morphological features of chromosome transfer clones in comparison with the parental cells, ROSE199, ROVE12, SKOV3 and MCF7. Similar morphological alterations were observed for cell lines NMU and C6. While the donor chromosome was retained in

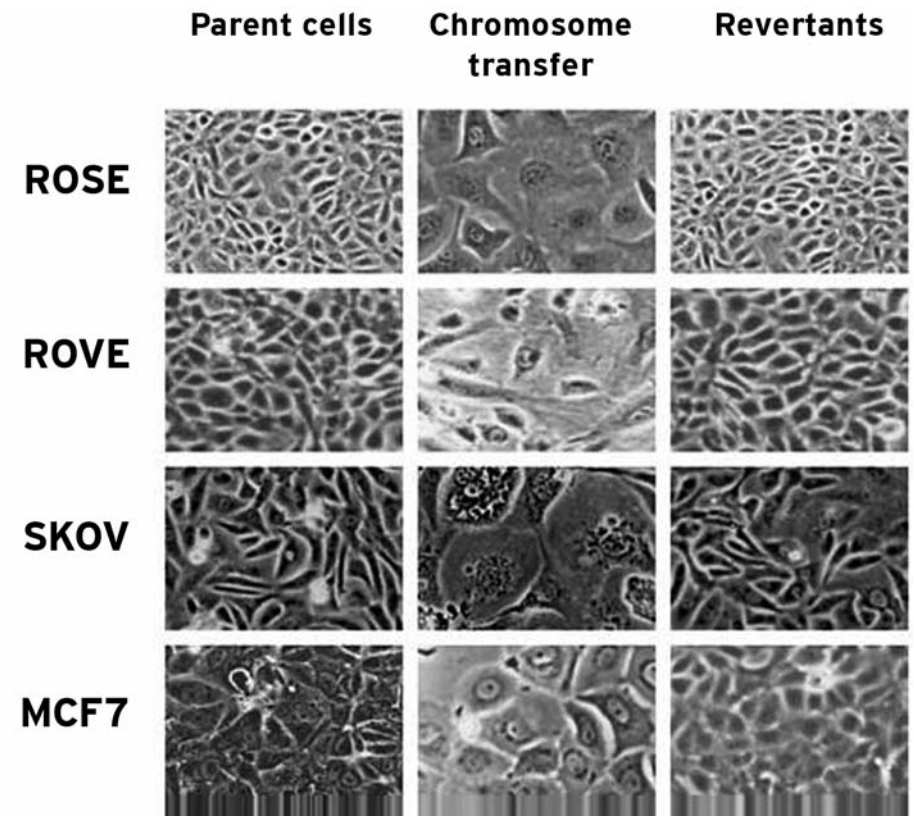

Figure 1. *Photomicrographs of rat ovarian tumor cell lines ROSE199 and ROVE12, human ovarian tumor cell line SKOV3 and human breast tumor cell line MCF7, before and after the transfer of chromosome 6. All cells were photographed and enlarged at identical magnifications.*

the microcell hybrids by selection, sporadic colonies of immortal revertant cells spontaneously arose among senescent cells (Figure 1). The reversion to immortal growth was likely due to the inactivation of the senescence gene by mutation, deletion or epigenetic gene silencing.

Mapping of SEN6A. A total of 20 revertant clones were isolated for cell lines ROSE199, ROVE12, NMU and C6, and 3 for SKOV3. In addition, one each mixed cell population of immortal revertants was created for ROSE199 and SKOV3 and three mixed populations for ROVE12, comprising cells from 6-10 colonies.

The independent revertant clones and mixed cell populations were analyzed for 152 DNA markers mapped at 6q13-21 (Figure 2A). Each revertant, ROSE/6R1, ROSE/6R2, ROSE/6R3, ROSE/6R4 and ROSE/6qR4 shown in Figure 2A, represents multiple clones containing identical deletions. The data revealed that each revertant retained the gpt tag (not shown) and a variable number of donor chromosome markers, but had lost one or more, usually contiguous, human markers except ROSE/6R1 (Figure 2A). The revertants represented by ROSE/6R1 did not show the deletion of any marker, whereas all the markers were deleted in a set of four revertant clones (data not shown). The revertants represented by ROSE/6R2, ROSE/6R3 and ROSE/6R4 showed deletions of multiple markers, while only a single expressed sequence tag (EST) marker was deleted in revertant ROSE/6qR4 (Figure 2A). The deletions of the markers in the same region were also observed in revertants NMR1 and NMR2 of mammary tumor cells and C6R1 of a rat glioblastoma. The deletions observed in four mixed cell populations, ROSE/6M1, ROVE/6R1, ROVE/6R2 and ROVE/6R3, were especially informative (Figure 2A). Since each mixed cell population was comprised of multiple clones, a deletion represented the loss of a marker shared among the constituent clones. Furthermore, deleted markers were contiguous in each mixed population. Interestingly, the single EST marker deleted in revertant ROSE/6qR4 was also deleted in the mixed cell population. The common deleted region, in the revertant clones, was flanked by markers D6S300 and D6S1028, which encompasses approximately 1cM genetic interval at 6q16.3 (Figure 2A).

Deletion mapping in revertants of the human SKOV3/6R cells was hindered by the lack of polymorphism between recipient and donor chromosome alleles for most of the

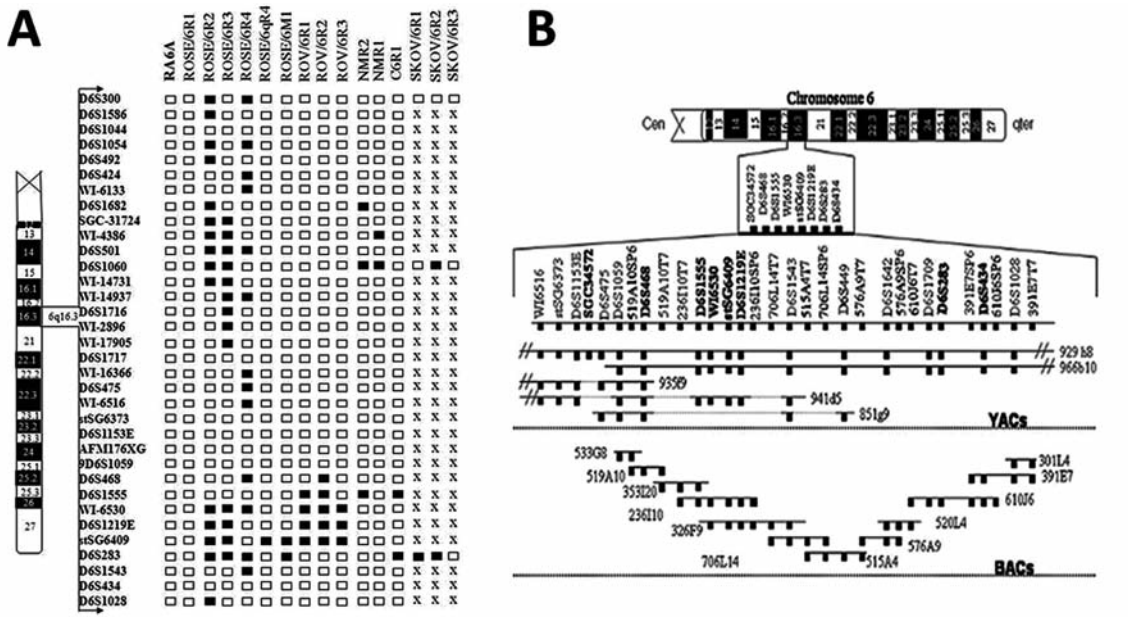

Figure 2. *A: Deletion analysis of chromosome 6 and 6q transferred into rat and human tumor cell lines. Partial chromosome 6 cytogenetic bands are shown with the interval q16.3 expanded. The polymorphic markers designated in the first column were amplified by PCR using the DNA from specific clones as template. RA6A and RA6q cell lines were used as source of donor chromosome. The presence of all markers in RA6A is shown, and these markers were also present in RA6q (not shown in the figure). ROSE199, ROVE12, NMU, C6 and SKOV3 are recipient cell lines. Suffixes R1, R2, R3 and R4 indicate the revertant clones. ROSE/6M1, ROV/6R1, ROV/6R2 and ROV/6R3 represent mixed cell populations of revertant clones. Filled rectangles indicate the absence of a specific marker, empty rectangles designate the presence of a specific marker and X indicates a noninformative marker. The noninformative markers were nonpolymorphic between donor chromosome and SKOV3 cells. B: Physical map of 6q16.3. The location of DNA markers, corresponding YAC and BAC clones spanning the SEN6A locus are indicated.*

markers. However, three markers D6S283, D6S1060 and D6S300, located in the vicinity of deletions observed in the rat cells, were polymorphic. Among these markers, D6S283 was deleted in SKOV/6R1, while both D6S283 and D6S1060 were deleted in SKOV/6R2. No deletions were detected in SKOV/6R3 (Figure 2A) and the mixed revertant cell population of SKOV/6 (data not shown).

The transfer of chromosome 6 from revertant ROSE/6qR4, which carried the smallest deletion, to SKOV3, MCF7, ROSE199 and ROVE12 cells failed to restore senescence in recipient cells (data not shown). These results further confirmed that a deletion observed in clone ROSE/6qR4 inactivated senescence inducing activity encoded by chromosome 6q.

Identification of YAC and BAC clones mapping to the SEN6A locus. Five YAC clones, 929h8, 966b10, 935f9, 941d5 and 851g9, each carrying at least one of the markers located in the consensus deletion, were identified through Human Genome database search, and obtained from Research Genetics (Huntsville, AL). The presence of specific marker sequences on these YAC clones was confirmed and a contiguous YAC map of 1 Mb region was assembled (Figure 2B). YACs 966b10 and 929h8 were confirmed to be non-chimeric and were mapped at 6q16.3 by fluorescence in situ hybridization (FISH, Figure 3A). Furthermore, marker analysis, and physical mapping of YAC 966b10 by FISH indicated it to contain a contiguous stretch of genomic DNA from 6q16.3 (Figure 3A). However, the FISH pattern of YAC clones 935f9, 941d5 and 851g9 revealed that these clones contained DNA segments from different chromosomes and were chimeric (data not shown).

Similarly, 35 bacterial artificial chromosome (BAC) clones were identified through repeated screening of a BAC library with the markers located in the vicinity of deletions in the revertant clones or by data base search. Individual BAC clones were analyzed for marker content by PCR, and overlapping clones were identified by using end-specific primers from the human insert in each BAC clone. These analyses led to the identification of 12 overlapping BAC clones representing the shortest tilling path at the SEN6A locus and confirmed the physical map derived from the contiguously located YAC clones (Figure 2B).

Functional testing of a YAC carrying SEN6A gene. YAC 966b10 was chosen for functional testing, and an unrelated YAC 813e was selected as a negative control. It is worth

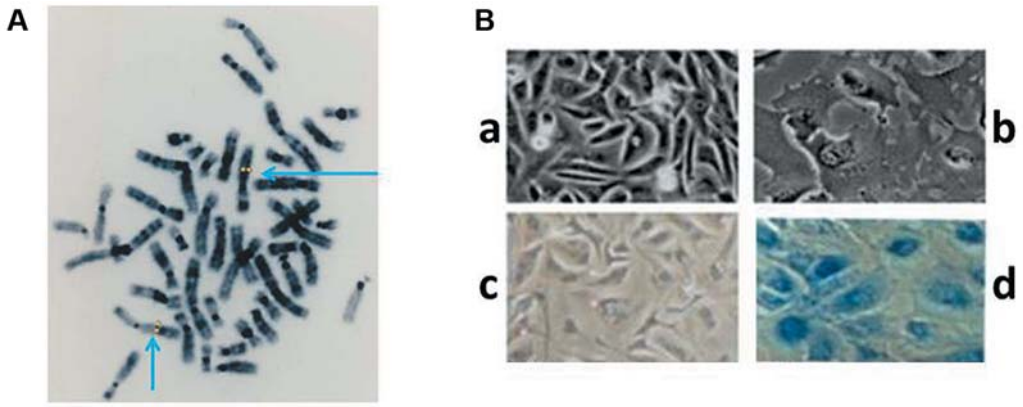

Figure 3. *A: Fluorescence in situ hybridization of YAC 966b10 to human metaphase chromosomes. YAC DNA was labeled with fluorescence tagged nucleotides and hybridized with metaphase spreads of human chromosomes. The fluorescence on hybridized chromosome was visualized and confirmed to be at chromosome 6q16.3. B: Morphology of SKOV3 cells before (a) and after (b) the transfer of YAC 966b10. SKOV3 cells show absence (c) and presence (d) of SA β-galactosidase activity, before and after YAC transfer respectively. All photomicrographs were taken at an identical magnification and different fields of view were then enlarged for presentation purposes.*

Table I. *Senescent colonies arising after transfer of YAC 966b10 into various cell lines.*

Transferred YAC	Recipient cells	Total colonies	Senescent colonies	Immortal colonies
966b10/A9	ROSE199	35	25	10
966b10/A9	ROVE12	17	12	5
966b10/A9	SKOV3	18	8	10
966b10/A9	MCF7	15	7	8
T966b10/A9	ROSE199	25	0	25
T966b10/A9	SKOV3	15	0	15
813/A9	ROSE199	8	0	8
813/A9	SKOV3	10	0	10

T: Truncated YAC966b10; 813: negative control.

mentioning that YAC 813e maps to a region that is unrelated to the senescence locus. Both YAC clones were retrofitted to incorporate neo gene for selection in mammalian cells. The size and the integrity of retrofitted YACs were ascertained by PFGE and Southern hybridization, and the presence of neo gene was confirmed by PCR (data not shown). Retrofitted derivative YAC clones 966neo3 and 813neo1 were introduced into rat (ROSE199 and ROVE12) and human (SKOV3 and MCF7) immortal cell lines.

The introduction of YAC966neo3 did not affect the proliferation of A9 cells as previously observed for A9 cells carrying the human chromosome 6. After confirming the presence of specific YAC clones in A9 cells, the biological significance of A9/966neo3 and A9/T966neo that contained an intact or a truncated YAC966b10 was characterized, respectively.

The YAC transfer colonies that survived in the G418 selection medium were examined at regular intervals to assess the morphology and growth characteristics. Based on these criteria, the colonies were classified as either immortal colonies that were indistinguishable from the parental recipient cells or senescent colonies that displayed characteristic enlarged, flattened and vacuolated cell morphology (Figure 3B). The transfer of 966neo3 to ROSE199, ROVE12, MCF7 and SKOV3 cells led to a senescence phenotype, similar to that observed following the transfer of chromosome 6 to these cells (Figure 1). However, the transfer of truncated YAC T966neo and unrelated YAC 813e showed no alteration in the parental cell phenotypes (Table I).

The senescent cells multiplied with an initial doubling time of 48-96 h, determined by cell count under the microscope, and progressively increased until cells entered the stage of complete growth arrest (data not shown). These senescent colonies consisted of approximately 500-2000 viable cells that remained attached to the surface for periods varying from 1 to 8 months. However, the immortal colonies multiplied continuously with doubling times of 16-20 h for rat and 24-30 h for human cells, respectively. At the end of the growth period, senescent colonies were analyzed by staining for SA- β-gal activity and

BrdU incorporation. The senescent colonies showed positive SA- β-gal activity (Figure 3B), and negative BrdU incorporation (data not shown), whereas immortal colonies and parental cells were negative for SA-ß-gal activity (Figure 3B).

Three senescent colonies of ROSE/966neo3 and two immortal isolates of the same cell line were analyzed for neo and human markers located on YAC 966b10. These analyses demonstrated the presence of neo and all 7 markers in senescent colonies, but part of the YAC was deleted in immortal colonies (data not shown). However, SKOV3 cells could not be analyzed for markers due to the lack of polymorphisms between donor YAC and recipient cells. It was noted that some undetermined amount of mouse A9 DNA was probably carried over during the transfer of YAC DNA from mouse A9 cells into recipient cells. However, the irrelevance of nonspecific transfer of mouse DNA for senescence was confirmed by using an unrelated YAC clone as a negative control.

After monitoring ROSE/966neo3 colonies for 4 weeks, two senescent colonies were transferred into a non-selective medium. In the absence of selection pressure, immortal cells proliferated due to the segregation of the chromosome bearing the integrated YAC. The analysis of immortal revertant colonies confirmed the loss of human markers. The reversion of senescent cells to immortal growth concurrent with the loss of human DNA showed that senescence was indeed related to the introduction of YAC 966b10. The transfer of a truncated derivative YAC present in clone T966neo/A9 and another YAC clone (823neo1) from an unrelated chromosomal region did not induce senescence in ROSE199 and SKOV3 cells.

Discussion

The experiments presented here underscore the significance of chromosome transfer as a functional strategy for gene cloning, illustrate the utility of revertant colonies for high resolution mapping of the SEN6A locus that led to the isolation of YAC clones for functional testing and describe a two-step gene transfer protocol for introducing YAC clones into mammalian cells. The presence of senescence activity in this region was confirmed by transfer of an 810 Kb YAC 966b10 into rat and human ovarian and breast tumor cells that led to growth arrest and senescence. Furthermore, marker analysis and physical mapping of YAC 966b10 by FISH revealed that it contained a contiguous stretch of genomic DNA from 6q16.3.

The presence of senescence/tumor suppressor genes on various human chromosomes is illustrated by restoration of senescence following the introduction of several individual chromosomes into transformed cells. In addition to SEN6A presented here, SEN6 has been mapped to 6q27 (28) and another locus has been suggested for the region 6q21 (29). The mutations or deletions in some of these genes may represent key events in the multi-step process of tumorigenesis. Interestingly, a plant homeodomain containing gene PHF10 (28) and ribonucleaseT2 (RNaseT2) gene (30, 31), both mapping to the SEN6 containing locus at 6q27, led to decreased proliferation of normal and SV40 immortalized human fibroblast cells, but not senescence. Furthermore these genes are not related to the cell cycle suppressor gene p21 that is located on 6p21. In light of these observations, we believe it is likely that a major senescence causing activity is associated with the SEN6A locus while the SEN6 locus on chromosomal interval 6q27 either has a senescence modifier function or codes for growth suppressor genes whose combined expression leads to a senescent phenotype.

The biological relevance of this chromosome in carcinogenesis is indicated by loss of heterozygosity for 6q markers in breast cancer, prostate cancer, hereditary mixed polyposis syndrome and childhood acute lymphoblastic leukemia (32-35). These reports suggest that in addition to SEN6, SEN6A and the 6p21 linked senescence genes; chromosome 6 is enriched with tumor suppressor and senescence genes. Given a limited number of relevant genes, such as protein tyrosine phosphatase receptor type K (PTPRK) and glutamate receptor (GluR6), located in this chromosomal interval, further studies to characterize these two genes for their roles in senescence and carcinogenesis are planned. Furthermore, it is important to note that p21, several growth suppressors and the major histocompatibility complex are located on chromosome 6.

In conclusion, YAC 966b10 encodes either the complete or a functionally active SEN6A gene that confers the senescent phenotype to immortal cells. The deletions of sequences from the YAC interrupt the SEN6A coding sequence and cause the senescent cells to revert back to an immortal phenotype. Thus the transfer of YAC or BAC clones could potentially lead to the accurate localization, identification and functional cloning of other important genes involved in tumorigenesis. These functional strategies could also be applied for cloning of other genes that impart specific phenotypes to cultured cells.

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