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Introduction of human chromosome 11 via microcell transfer controls tumorigenic expression of HeLa cells

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Both tumorigenic segregant HeLa × human fibroblast hybrids and tumorigenic HeLa (D98/AH-2) cells can be converted to a non-tumorigenic state following introduction of a single copy of a fibroblast t(X;11) chromosome. The translocated chromosome contains ~95% of the 11 chromosome and the q26–qter portion of the X chromosome which contains the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene. Introduction of a human X chromosome has no effect on tumorigenic expression. Suppression of tumorigenicity is relieved by selecting cells which have lost the t(X;11) chromosome by growth in medium containing 6-thioguanine (6-TG). Further, reintroduction of the t(X;11) chromosome into tumorigenic 6TG^R cells again suppresses tumorigenicity. Thus, the introduction of a single copy of a human chromosome 11 is sufficient to completely suppress the tumorigenic phenotype of HeLa cells and is suggestive of the presence of tumor-suppressor gene(s) on this chromosome.

Key words: suppression/tumorigenicity/microcell transfer

Introduction

Harris and colleagues (1969) first observed that fusion of highly tumorigenic mouse cells to mouse A9 cells of low tumorigenicity or normal mouse fibroblasts (Weiner *et al.*, 1971) resulted in hybrids with low tumorigenic potential. These early results were confirmed by other studies using both intraspecific and interspecific hybrid systems, leading to the suggestion that tumorigenicity behaves as a recessive trait. In other studies, that utilized intraspecies rodent hybrid cells or interspecies human × rodent hybrid cells (where rapid unidirectional segregation of human chromosomes occurs), the conclusions drawn were that tumorigenicity behaves as a dominant trait (Barski *et al.*, 1961; Croce, 1984). Support for the observation of suppression of tumorigenicity came from studies of intraspecies human cell hybrids. Stanbridge (1976) constructed karyotypically stable intraspecific human/human cell hybrids from tumorigenic HeLa cells and normal diploid human fibroblasts. These hybrids retained the transformed characteristics of the HeLa parent but were completely suppressed for the tumorigenic phenotype. Only after prolonged passage in culture did rare tumorigenic segregants arise. Karyotype analysis of the non-tumorigenic hybrids and the resulting tumorigenic segregants showed a statistical correlation between loss, presumably via chromosomal non-disjunction, of a single copy of chromosomes 11 and 14 and the reappearance of tumorigeniciity in these rare segregants (Stanbridge *et al.*, 1981).

Independent cytogenetic analyses by Klinger (1982) confirmed the association of the loss of a single copy of chromosome 11 with re-expression of tumorigenicity but also implicated a number of other chromosomes. This association of loss of chromosome 11 with re-expression of tumorigenicity was only tentative because it was impossible, using conventional banding techniques, to identify the parental origin of the segregated chromosome. A more refined analysis that allowed such an identification to be made became possible with the advent of restriction fragment length polymorphism (RFLP) probes. Both Srivatsan *et al.* (1986) and Kaelbling and Klinger (1986) have shown that it is the normal fibroblast copy of chromosome 11 that is lost from tumorigenic segregants using RFLP analysis. Srivatsan *et al.* further showed that loss of fibroblast chromosome 14 is not associated with tumorigenic re-expression. Thus, these studies suggest that loss of a single copy of a fibroblast chromosome 11 is sufficient to allow re-expression of tumorigenicity in these HeLa × fibroblast hybrids.

We show here direct proof for the involvement of chromosome 11 in tumorigenic suppression of HeLa-derived hybrids by the transfer of a single copy of a fibroblast t(X;11) chromosome to a tumorigenic HeLa × fibroblast recipient cell, resulting in a non-tumorigenic hybrid. Reversibility of such suppression can be demonstrated by selection in 6-thioguanine (6-TG) for segregants which have lost the t(X;11) chromosome. These segregants are as tumorigenic as the original hybrid cell. Finally, it is shown that these tumorigenic segregants can again be suppressed for tumorigenicity by re-introduction of the t(X;11) chromosome. The same phenomenon of suppression of tumorigenicity is also seen when the t(X;11) chromosome is introduced into HeLa cells.

Results

The t(X; 11) × A9 hybrid

The t(X;11) balanced translocation chromosome found in the human fibroblast line GM3552 is a chromosome 11 whose q arm terminus has been replaced by the Xq26–Xqter portion of the X chromosome, which includes the hypoxanthine guanine phosphoribosyl transferase (HPRT) locus, enabling cells which contain the t(X;11) to survive in HAT (hypoxanthine/aminopterin/thymidine) selective medium (Scott *et al.*, 1979). Initial attempts in this laboratory to transfer the t(X;11) directly from GM3552 fibroblasts to HPRT-deficient ESH-15 T1 cells via microcell transfer were unsuccessful because it was difficult to obtain sufficient numbers of microcells directly from human fibroblasts, an observation made by others (McNeill and Brown, 1980). Rodent cells and rodent–human hybrids, on the other hand, are very efficient in forming microcells. Therefore, GM3552 fibroblasts were first fused to mouse A9 cells and the resulting mouse–human somatic cell hybrids, intended for use as microcell donors, were selected for growth in HAT medium. Fortuitously, after prolonged passage in HAT medium, one such hybrid, 110.1, was found to be have segregated all human chromosomes

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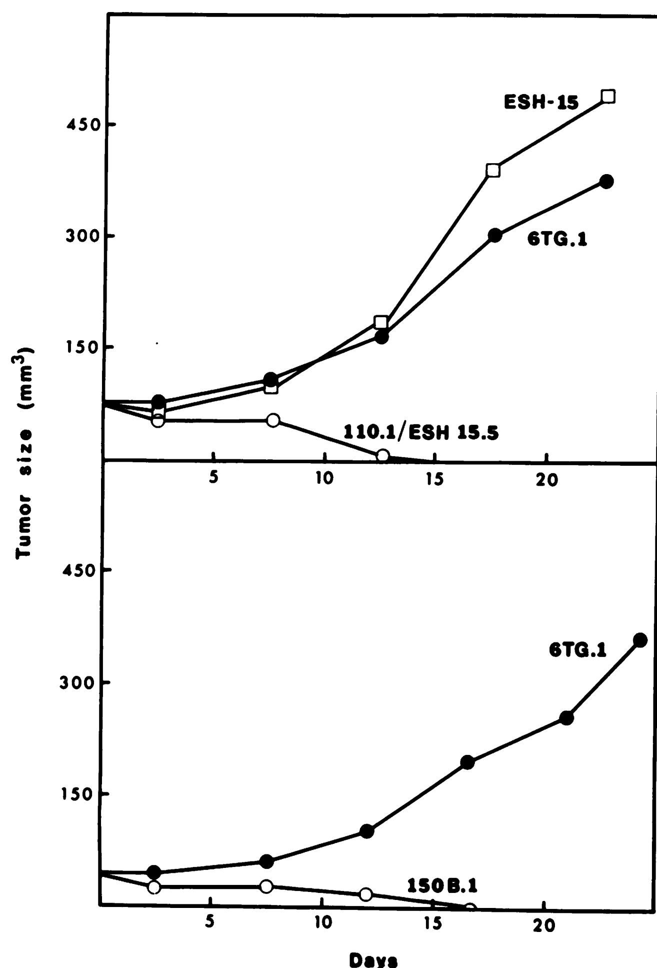

Fig. 1. Tumorigenicity of selected HeLa × fibroblast hybrids. In each case 1 × 10^7 cells were inoculated s.c. and tumor masses were measured periodically over a 25-day period. **Upper panel**: ESH15 = tumorigenic HeLa × fibroblast hybrid; 110.1/ESH 15.5 = ESH15 containing the t(X;11) chromosome; 6TG.1 = 110.1/ESH15.5 back-selected in medium containing 6-TG to select for cells that have lost the t(X;11) chromosome. **Lower panel**: 150B.1 = 6TG-1 hybrid into which the t(X;11) chromosome has again been transferred; 6TG-1 = same as in upper panel.

except for the t(X;11) and thus provided an ideal source of the t(X;11) for subsequent microcell transfers.

Suppression of a tumorigenic whole cell hybrid

The hybrid line ESH-15 T1 is a 6-TG-resistant tumorigenic segregant isolated previously in this laboratory (Weissman and Stanbridge, 1980) from a non-tumorigenic somatic cell hybrid, ESH-15, which originally resulted from a fusion between tumorigenic D98/AH-2 (HeLa) cells and normal human fibroblasts. Because of its highly tumorigenic phenotype and its resistance to 6-TG, ESH-15 T1 was used as the recipient for a number of microcell-mediated chromosome transfers in an attempt to suppress its tumorigenic phenotype with a copy of chromosome 11 from normal human fibroblasts.

Microcell-mediated transfer of the t(X;11) to ESH-15 T1 resulted in the appearance of HAT-resistant colonies with a typical frequency of 2 × 10^{-5}. Morphologically, the individual cells in these microcell hybrid colonies had a larger diameter than either parent and tended to grow more slowly than either parent. Five hybrid colonies from separate HAT selection dishes were isolated and expanded for further analysis, including chromosome counts, RFLP analysis to confirm the presence of the t(X;11) in the microcell hybrids and tumorigenicity assays. Also, all hybrids were tested for the presence of any mouse chromosomes, intact or fragmented, which may have been accidentally transferred during the microcell transfer process. Any such contaminated hybrids were removed from the study. DNA extracted from the hybrid cells that had received t(X;11) were also hybridized to genomic mouse DNA in order to detect any contaminating mouse DNA. None of the microcell hybrids described in this study contained mouse DNA (data not shown).

Tumorigenicity assays

1 × 10^7 cells from each of the hybrid clones were inoculated subcutaneously into 6-week-old athymic nude mice. Figure 1 shows tumor growth rates for selected microcell hybrids. For all five clones, the hybrid cells formed palpable nodules which increased in size slightly for the first few days, then steadily decreased in size until they had completely regressed by 20 days post-inoculation. By comparison, the original ESH-15 T1 cells formed tumors which increased in mass steadily during the entire course of the experiment (Figure 1 and Table I).

Three of the five suppressed microcell hybrids were subsequently grown in 6-TG (8 μg/ml) to select for 6-TG-resistant segregants which had lost the t(X;11) chromosome. Three such 6-TG-resistant segregants were isolated and expanded in order to test whether the suppressed hybrids could re-express the tumorigenic phenotype upon loss of the t(X;11). Figure 1 shows the resulting tumor formation for a 6-TG-resistant clone (6TG.1) which was typical for all the 6-TG-resistant clones tested (Table I). The growth rate of the 6-TG-resistant segregants was slightly slower than for the parental ESH-15-T1 control but eventually the resulting tumors reached the same relative size as the control after 60 days.

A secondary microcell transfer of the t(X;11) was made into the now tumorigenic 6-TG-resistant segregants. This was done to explore the possibility that during back-selection in medium containing 6-TG the suppressed hybrids segregated out essential chromosomes other than t(X;11), which were responsible for the reappearance of tumorigenicity. Three such microcell hybrids with a re-introduced t(X;11) were inoculated into nude mice and found to be as completely suppressed for tumorigenicity as the original microcell hybrids. Figure 1b shows the growth curve for a typical secondary microcell hybrid (150B.1). The results of the tumorigenicity assays for all of these microcell hybrids are summarized in Table I.

In order to assign the tumor suppression activity to only the chromosome 11 portion of the t(X;11) and to exclude the possibility that such suppression activity resided on the Xq26−Xqter portion, a separate series of microcell transfers was made into ESH-15 T1 using a mouse−human microcell hybrid (MCH200-9), which contains an intact human X as the only human chromosome represented in the hybrid as the microcell donor (M.Layton and E.J.Stanbridge, unpublished observations). Microcell hybrids isolated from this fusion were found to be as tumorigenic as the parental ESH-15 T1 (Table I). Thus, the Xq26−Xqter portion of the t(X;11) chromosome can be ruled out as containing the tumor suppressor locus.

RFLP analysis

In order to detect the presence or absence of the t(X;11) chromosome in the various microcell hybrids and 6-TG segregants, the c-Ha-*ras* oncogene probe (6.6-kb fragment) (Shih and Weinberg, 1982) was used to detect a polymorphic *Taq*I site on the p arm of chromosome 11. Thus, in the Southern blot illustrated in Figure 2, while all cell lines show a common 2.3-kb band, the

Table I. Tumorigenicity testing of HeLa × fibroblast hybrid cells

Hybrid cell designation	Growth in selective medium		Chromosome count (mode)	Presence of translocated X;11 chromosome	Tumorigenicity assay no. tumors/no. inoc.
	HAT	6-TG			
Parental ESH 15 T1	−	+	94	−	4/4
t(X;11) transferred into ESHT1					
110.1/ESH15.1	+	−	93	+	0/3
110.1/ESH15.2	+	−	94	+	0/3
110.1/ESH15.4	+	−	N.D.	+	0/3
110.1/ESH15.5	+	−	90	+	0/3
110.1/ESH15.6	+	−	N.D.	+	0/3
110.1/ESH15 6-TG-resistant segregants					
110.1/ESH15.2 6-TG.1	−	+	88	−	3/3
110.1/ESH15.5 6-TG.1	−	+	89	−	3/3
Re-introduced t(X;11)					
150B.1	+	−	90	+	0/3
150B.3	+	−	90	+	0/3
150B.4	+	−	90	+	0/3
X chromosome transferred to ESH15T1					
200-9/ESH−5.1	+	−	N.D.	−	3/3
200-9/ESH15.4	+	−	N.D.	−	2/3
200-9/ESH15.7	+	−	N.D.	−	3/3

N.D. = Not determined.

t(X;11) in 110.1 contains an RFLP allele represented by a single 2.5-kb band, while the ESH-15 T1 parent contains heterozygous RFLP alleles for c-Ha-*ras* on chromosome 11 which are seen as 3.0- and 3.4-kb bands. In previous studies, we have shown that the 3.0-kb band corresponds to homozygous RFLP alleles of HeLa and the 3.4-kb band corresponds to a fibroblast RFLP allele (E.S.Srivatsan *et al.*, 1986; E.J.Srivatsan and Stanbridge, unpublished observations). The microcell hybrid 110.1/ESH-15 contains the 2.5-kb t(X;11) allele plus the ESH-15 T1 RFLP alleles, while the resulting 6-TG-resistant segregants have lost only the t(X;11) allele. Tumors which arose from inoculated 6-TG-resistant segregants were removed from the animals and re-established in culture. The DNAs from these tumors (TR1 and TR2) are, as expected, missing the t(X;11) allele (Figure 3). Finally, when the t(X;11) was re-introduced into the 6-TG-resistant segregants via microcell-mediated transfer, the characteristic t(X;11) 2.5-kb band re-appears. These results confirm the successful transfer of the selectable t(X;11) chromosome to and loss from the different recipient cells.

Chromosome distribution

Metaphase spreads of the microcell hybrids were examined and histograms of the chromosome counts were generated in order to detect significant shifts in total chromosome number during microcell transfer and back-selection on 6-TG. Figure 4 shows a histogram comparison of chromosomes in the initial microcell hybrid 110.1/ESH-15.5, the resulting 6-TG-resistant segregant and the secondary microcell hybrid with a re-introduced t(X;11) chromosome. While the appearance or loss of a single chromosome, presumably the t(X;11), in the different hybrids is not obvious in this comparison, no large shifts in chromosome number occurred during microcell transfer and 6-TG back-selection.

Suppression of tumorigenicity in D98/AH-2 cells

The foregoing experiments, as well as the RFLP data published previously (Srivatsan *et al.*, 1986; Kaelbling and Klinger, 1986)

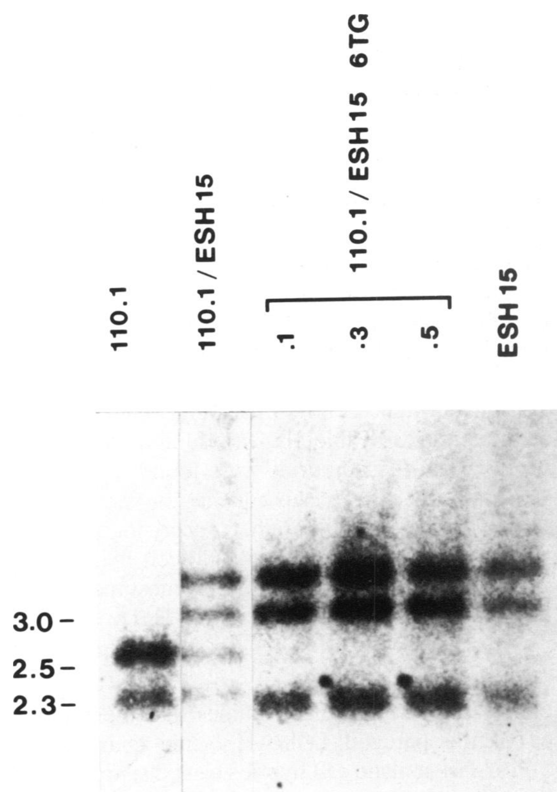

Fig. 2. RFLP analysis to detect the presence or absence of the t(X;11) chromosome. See text for experimental details. *Taq*I-digested DNA was hybridized to a c-Ha-*ras* probe (maps to the p arm of chromosome 11). 110.1 = A9 mouse cell line containing the t(X;11) chromosome as the only human chromosome. The RFLP fragment specific for this chromosome is the 2.5-kb band. This band is absent in the original ESH15, present in the 110.1/ESH15 and again absent in the back-selected 110.1/ESH15 6TG clones .1, .3 and .5.

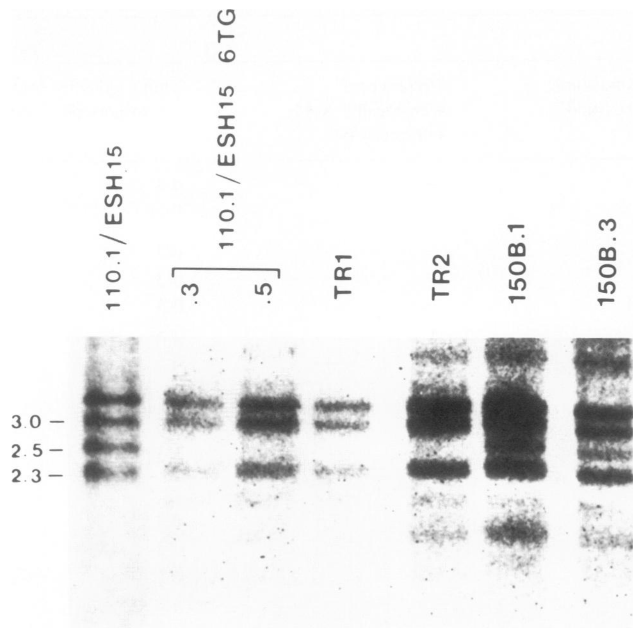

Fig. 3. RFLP analysis of hybrids and tumor reconstitutes. The same protocol was used as in Figure 2. Panels TR1 and TR2 are DNAs extracted from cell cultures of tumors derived from 110.1 ESH15 6TG.3. As expected, the 2.5-kb band is absent. Panels 150B.1 and 150B.3 are DNAs of the hybrid cell lines into which the t(X;11) chromosome has been re-introduced. They are non-tumorigenic (see Table I).

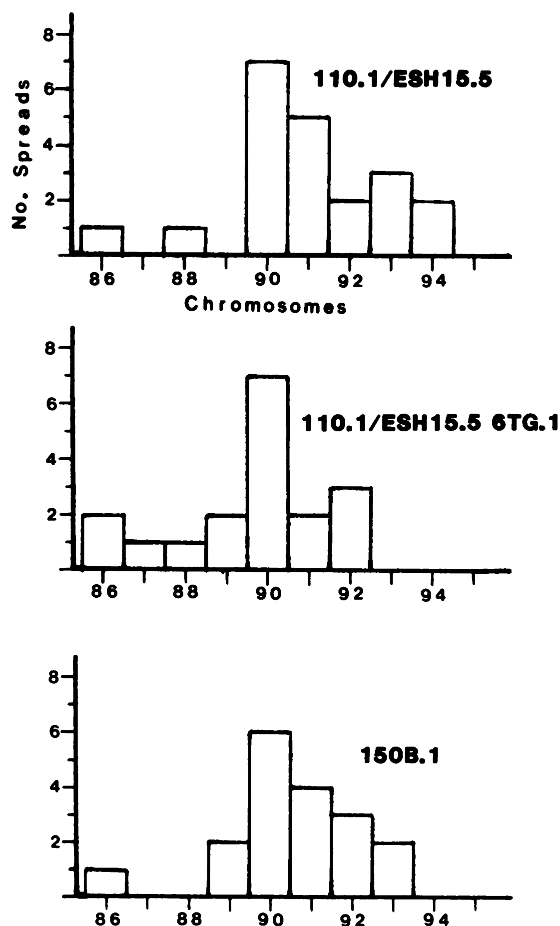

Fig. 4. Histograms illustrating the chromosome distributions of the HeLa × fibroblast hybrids containing or lacking the t(X;11) chromosome after selection in HAT or 6-TG media. See Figure 1 for cell line identification.

would suggest that the presence of two copies of normal fibroblast chromosome 11 (possibly in concert with other normal chromosomes) is necessary in order to achieve complete suppression of the tumorigenic phenotype.

In order to determine whether a single copy of the fibroblast-derived t(X;11) chromosome in the absence of any other fibroblast-derived chromosomes (such as found in the ESH-15 hybrid) is, in fact, sufficient to suppress the tumorigenic phenotype of HeLa, the t(X;11) chromosome was introduced into the D98/AH-2 tumorigenic parent itself via microcell transfer. Of eight microcell hybrids isolated from a microcell transfer, four were expanded and examined for suppression of the tumorigenic phenotype. All four microcell hybrids were found to have been completely suppressed (Table II). In addition, 6-TG-resistant segregants of two of these hybrids were found to have lost the t(X;11) chromosome with a subsequent re-expression of the tumorigenic phenotype. Thus, rather surprisingly, given the results with the HeLa × fibroblast whole cell hybrids, the introduction of a single copy of normal chromosome 11 is sufficient to control tumorigenic expression of HeLa cells.

Discussion

We have shown clearly in these studies that the tumorigenic phenotype of the parental D98/AH-2 line and tumorigenic HeLa × fibroblast somatic cell hybrids can be suppressed by the addition, via microcell transfer, of a single copy of a fibroblast-derived translocated chromosome t(X;11). Furthermore, the tumor suppressor activity resides on the chromosome 11 portion and not the X chromosome portion of the chromosome. These results clarify the results from our earlier cytogenetic studies of somatic cell hybrids (Stanbridge *et al.*, 1981), which showed a statistical correlation between the loss of both a single copy of chromosome 11 and 14 and the reappearance of tumorigenicity in somatic cell hybrids which were initially non-tumorigenic. In the case reported here, the direct addition of a single copy of the t(X;11) chromosome is sufficient to suppress the tumorigenic phenotype of D98/AH-2 and the tumorigenic segregant hybrid ESH-15 T1. Likewise, subsequent removal of the t(X;11) from the suppressed ESH-15 by back-selection in 6-TG allows re-expression of the tumorigenic phenotype. The reversible nature of the tumorigenic phenotype is demonstrated by once again suppressing the tumorigenic phenotype by re-introducing a copy of the t(X;11) into the tumorigenic 6-TG-resistant segregants.

Suppression of tumor formation by the HeLa × fibroblast hybrid ESH15 (T1) by introduction of a single copy of chromosome 11 might be expected to occur because our previous studies had shown that tumorigenic re-expression in HeLa × fibroblast hybrids is accompanied by loss of a single copy of chromosome 11. Therefore, the microcell transfer effected a simple replacement of the putative lost chromosome. However, it was unexpected that a single copy of the t(X;11) chromosome, in the absence of any other fibroblast chromosomes, would be sufficient to suppress the tumorigenic phenotype of the parental D98/AH-2, since previous RFLP studies on whole cell hybrids showed that tumorigenic segregants of the whole cell hybrids usually retained one homologue of the fibroblast chromosome 11. This raises the possibility that genes on other fibroblast-derived chromosomes may in some way modulate the level of expression of the chromosome 11-specific suppression activity. Therefore, in the absence of any other fibroblast-derived

Table II. Tumorigenicity testing of HeLa × microcell hybrids

Cell designation	Growth in selective medium		Chromosome count (mode)	Presence of translocated X;11 chromosome	Tumorigenicity assay no. tumors/no. inoc.
	HAT	6-TG			
Parental					
D98/AH-2	−	+	62	−	4/4
t(X;11) transferred into D98/AH-2					
110.1/D98.1	+	−	63	+	0/3
110.1/D98.2	+	−	63	+	0/3
110.1/D98.4	+	−	63	+	0/3
110.1/D98.7	+	−	N.D.	+	0/3
110.1/D98 6-TG-resistant segregants					
110.1/D98.1 6TG-1	−	+	62	−	3/3
110.1/D98.2 6TG.1	−	+	63	−	3/3

N.D. = Not determined.

chromosomes, the chromosome 11-specific suppressor gene(s) may be expressed constitutively at a higher level and a single copy would then be able to sustain suppression of the tumorigenic phenotype.

The cytogenetic analyses of HeLa × fibroblast hybrid cells by Klinger (1982) have, like those of ourselves (Stanbridge *et al.*, 1981), implicated chromosome 11 as playing a role in tumor suppression and have also implicated other chromosomes, including numbers 1, 2, 16, 17, 19 and 21. The data presented here clearly indicate that the introduction of a single copy of normal fibroblast chromosome 11 into HeLa is sufficient to suppress tumorigenicity. Whether any of the other chromosomes identified by Klinger are capable of such suppression remains to be seen. Transfer of the chromosome 11 in these studies was facilitated by the natural t(X;11) translocation, which resulted in the transfer of the selectable marker HPRT to this autosome and thus allowed for selection of cells containing the t(X;11) chromosome (in HAT medium) or for cells that had lost it (in 6-TG medium). Very few chromosomes contain genetic markers that can be used for dominant selection. We have, therefore (Saxon *et al.*, 1985) developed strategies for integrating dominant selectable markers into representative chromosomes of the entire human karyotype. This will facilitate selective microcell transfer of any single human chromosome to a recipient cell, thereby allowing us to determine if other human chromosomes carry genetic loci capable of suppressing the tumorigenic potential of HeLa and other malignant human cells.

The finding that a single chromosome is implicated in the control of tumorigenic expression in HeLa cells has a parallel in rodent cells. In an extensive cytogenetic analysis of a series of intraspecies rodent hybrid cells, Evans and colleagues (1982) found that normal chromosome 4 was implicated in suppression of malignancy of a number of different mouse tumor cells.

Further refinements currently in progress include using fragments of human chromosomes to more finely map the location of the tumor suppressor loci and eventually clone the gene(s) responsible for tumor suppression.

As has been suggested for retinoblastoma and the Wilm's tumor/aniridia syndrome, both of which are associated with a specific interstitial chromosome deletion (Knudson, 1985), we suggest that the putative tumor suppressor gene(s) (Stanbridge, 1985) which resides on the normal copy of chromosome 11 is another example of dominantly acting genes which control cells from entering a neoplastic state. Thus, as in the case of retinoblastoma and Wilm's tumor (Murphree and Benedict, 1984; Koufos *et al.*, 1984), neoplastic expression of HeLa may be associated with homozygous deletion or alteration of the autosomal gene associated with control of tumorigenic expression. Of interest in this regard is the recent report of Kaelbling *et al.* (1986) who suggest a loss of heterozygosity for chromosome 11 in HeLa cells and that the two copies of chromosome 11 found in those cells are both copies of one of the original homologs.

Although the mechanism for genetic control of tumorigenic expression is still unknown, histological examination of human cell hybrids which are suppressed for tumorigenicity suggest that the hybrid cells *in vivo* undergo terminal differentiation, and take on the phenotypic characteristics of the normal parental cell (Stanbridge *et al.*, 1983; Harris, 1985). It remains to be seen if HeLa cells that receive only a single copy of fibroblast chromosome 11 also differentiate *in vivo*.

Materials and methods

Cell lines and culture conditions

Human fibroblast line GM3552 containing the X;11 translocation was obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The HPRT-deficient HeLa cell line D98/AH-2 has been described previously (Weissman and Stanbridge, 1980). EHS-15 (T1) was previously isolated in this laboratory, and it is also HPRT deficient. $HPRT^-$ mouse A9 cells were a gift from Dr Keith Fournier. The other hybrid cell lines used in this study were isolated in this laboratory during the course of this study. All cell lines were maintained on Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum plus 100 IU/ml penicillin/streptomycin/fungizone. All cell lines were regularly monitored for the presence of mycoplasmas (Stanbridge, 1981) and were always found to be negative.

RFLP analysis

RFLP analysis of cell hybrid gneomic DNA, including DNA isolation, Southern transfers, DNA radiolabeling and restriction endonuclease digestions were carried out as previously described (Srivatsan *et al.*, 1986).

Microcell-mediated chromosome transfer and somatic cell hybridization

All microcell-mediated chromosome transfers were performed as previously described (Saxon *et al.*, 1985). Briefly, A9 cell lines containing single human chromosomes were treated for 48 h with 0.02 μg/ml colcemid (Calbiochem). Micronuclei were harvested by filling the flasks with medium containing 10 μg/ml cytochalasin-B (Sigma) and then centrifuging the entire culture flasks (25 cm^2, Nunc) in a fixed-angle rotor (Beckman JA-14) at 25 000 *g* for 65 min at 34°C. Microcell pellets were resuspended, filtered through 8 μm and 5 μm polycarbonate filters (Nucleopore) in series to remove large microcells and contaminating whole cells and karyoplasts. The filtered microcells were attached to recipient cell monolayers with 50 μg/ml phytohemagglutinin-P (PHA-P), then fused to recipient cells with PEG-1000 MW (Baker), 48% in MEM for 60 s.

All cell fusions were done as previously described (Saxon *et al.*, 1985).

Tumorigenicity assays

Cells to be assayed were harvested by trypsinization and suspended in serum-free MEM. Suspensions (0.2 ml) containing 1×10^7 cells were inoculated s.c. into congenitally athymic nu/nu (nude) mice. Animals were examined for presence of tumor formation at regular intervals and the dimensions of any resulting tumors were recorded.

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