The data further suggest that a copepod would assimilate almost twice as much protein by ingesting a stationary-phase cell as it would by ingesting a log-phase cell. Significantly higher assimilation efficiencies for Zn and S were observed in animals feeding on stationary-phase cells than on actively dividing cells, in close agreement with the relative quantities of these elements and protein in the cytoplasm of the cells (Fig. 1 and Tables 1 and 2). Copepods grazing on algal cells toward the end of a bloom could therefore be expected to assimilate more protein and protein-associated elements than those grazing on equal amounts of midbloom phytoplankton cells.

The class B and borderline metals (Ag, Cd, and Zn), which have greater affinities for S than N than O (*16*), showed greater penetration into the algal cytoplasm and therefore greater assimilation in grazers than did class A metals [Am (*10*) and our data; Th and Pu (*17, 18*)], which have greater affinities for O than N than S (*16*). The assimilation efficiencies that we measured for animals that ate stationary-phase diatoms are virtually identical to those measured for Cd (29.6%), Zn (48.4%), and Am (4.5%) in a separate study in which the copepod *Anomalocera patersoni* was fed stationary-phase *Isochrysis galbana* cells (*18*). The similar results suggest that our conclusions apply to other copepods feeding on nondiatom phytoplankton cells. Those elements that penetrate into the cytoplasm of phytoplankton and are assimilated by zooplankton thus enter the organic cycle in the sea, and through recycling, have longer oceanic residence times (*2*), consistent with the inverse relations observed between element residence times and enrichment factors in zooplankton fecal pellets (*19*). The results also explain why zooplankton fecal pellets are more enriched in particle-reactive nonessential elements than are the animals that produced them or the algal food from which they were produced (*1*).

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4. ^{110m}Ag was taken from a solution in 0.1*N* HNO_3 (added to cultures to give 74 kBq $liter^{-1}$, 21 nM); ^{241}Am was in 3*N* HNO_3 (in different experiments, a range of 18.5 to 37 kBq l^{-1}, 0.6 to 1.2 nM, was used); ^{14}C was in $NaH^{14}CO_3$ in distilled water (98.8 kBq $liter^{-1}$); ^{109}Cd was added in 0.1*N* HCl (296 kBq $liter^{-1}$, 9.3 nM); ^{32}P was in the form $NaH_2{}^{32}PO_4$ in distilled water (148 kBq $liter^{-1}$, 4 nM); ^{35}S was in the form $Na_2{}^{35}SO_4$ in distilled water (3.18 MBq $liter^{-1}$); ^{75}Se was in 0.5*N* HCl (in different experiments, a range of 37 to 660 kBq $liter^{-1}$, 0.136 to 2.42 nM selenite, was used); ^{65}Zn was in 0.1*N* HCl (296 kBq $liter^{-1}$, 0.18 nM). All acids were Ultrex. Radiotracers were added in microliter amounts; the pH of the cultures was adjusted to 8.0.
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Senescence of Nickel-Transformed Cells by an X Chromosome: Possible Epigenetic Control

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Transfer of a normal Chinese hamster X chromosome (carried in a mouse A9 donor cell line) to a nickel-transformed Chinese hamster cell line with an Xq chromosome deletion resulted in senescense of these previously immortal cells. At early passages of the A9/CX donor cells, the hamster X chromosome was highly active, inducing senescence in 100% of the colonies obtained after its transfer into the nickel-transformed cells. However, senescence was reduced to 50% when Chinese hamster X chromosomes were transferred from later passage A9 cells. Full senescing activity of the intact hamster X chromosome was restored by treatment of the donor mouse cells with 5-azacytidine, which induced demethylation of DNA. These results suggest that a senescence gene or genes, which may be located on the Chinese hamster X chromosome, can be regulated by DNA methylation, and that escape from senescence and possibly loss of tumor suppressor gene activity can occur by epigenetic mechanisms.

NEOPLASTIC TRANSFORMATION IS known to be a multistep process (*1*). One class of genes implicated in this process are tumor suppressor genes that in normal cells are thought to suppress the expression of transformation, malignancy, or metastatic progression and must either be lost, inactivated, or mutated in tu-

mor cells (*2, 3*). One mechanism by which tumor suppression may occur is via cellular senescence, the process that limits the lifespan of normal cells (*3*). Tumorigenic cells in culture are generally immortal and have overcome terminal senescence. In somatic cell fusion experiments (*4*), senescence has been shown to be dominant over immortality. Human chromosome 1 carries a gene that may be involved in normal cellular senescence (*5*), but a family of at least four suppressor genes is believed to exist (*4*); the chromosomal locations of these genes remains to be established.

Carcinogenic Ni compounds are inactive or weak in most gene mutation assays but induce damage primarily in the heterochromatic regions of chromosomes (*6*). We have demonstrated that treatment of Chinese hamster embryo (CHE) cells with Ni induced a high recovery of transformants that exhibited nonrandom deletions of the heterochromatic long (q) arm of the X chromosome (*7*). The frequency of Ni-induced transformation was significantly higher in male than female cells (*7*). It was thus hypothesized that the loss of an X chromosome tumor suppressor function was associated with Ni-induced transformation.

Normal CHE cells could not be used directly as X chromosome donors in a microcell-mediated transfer protocol because they did not readily form micronuclei. To generate Chinese hamster X chromosome donor cells, we fused male CHE cells with hypoxanthine-guanine phosphoribosyl transferase–deficient ($HPRT^-$) mouse A9 cells, which were very efficient in microcell formation (*8*). The retention of the hamster X chromosome in resulting hamster-mouse hybrids was accomplished by growth of the hybrids in HAT (hypoxanthine-amethopterin-thymidine) medium, which selected specifically for the functional *hprt* gene. In Chinese hamsters, the *hprt* locus is located on the short (p) arm of the X chromosome (*9*). The Chinese hamster X chromosome was transferred into A9 cells by microcell-mediated chromosome transfer and two cell hybrids, A9-CX clones 1 and 2, were obtained; an intact Chinese hamster X chromosome in the mouse cells was identified in each of these microcell hybrids by both C- and G-banding.

The intact Chinese hamster X chromosome from the A9-CX donor clones was transferred to the recipient $HPRT^-$ Ni-transformed Chinese hamster line Ni-2/TG^R (Table 1). Parental Ni-2 cells (*7*) were immortal, morphologically transformed, anchorage-independent, tumorigenic in nude mice, and exhibited a complete deletion of Xq, such that only the short arm of the X chromosome (Xp) was retained. Chromosome transfer was accomplished with a modified microcell-fusion procedure (*8, 10*) that permited the selective introduction of single chromosomes into recipient cells. A total of 76 HAT-resistant hybrids was obtained in ten independent fusion experiments with both A9-CX donors. Of these, 80% (60 clones) senesced with characteristic senescent colony morphology after reaching a size of several hundred to more than 1000 cells. Similar to Hayflick and Moorehead's original description of senescing cultures (*11*), individual senescing colonies ceased proliferation in situ, exhibiting flat and enlarged cell morphology (Fig. 1). Giant cells, which were visible 10 to 15 days after fusion, were usually the first indicators that a previously proliferating hybrid colony was beginning to senesce. Multinucleated cells also began to arise in senescing colonies. Senescing clones varied in final size from 25 to 1000 cells per colony and stopped dividing, but remained apparently alive and anchored to the plastic for up to 30 to 45 days. Occasionally, small senescent colonies (<50 cells) were observed to be nonproliferating for up to 20 days, and several eventually became detached from the dish. Growth stimulation of senescing colonies was not evident with fresh serum supplementation, which was provided every 3 to 4 days for the duration of the experiment. In general, senescing hybrid colonies could not be isolated from the experimental plate and therefore could not be grown into mass cultures. On two occasions, large hybrid colonies were isolated, but both senesced in culture after one passage and could not be propogated. In contrast to senescing colonies, nonsenescing hybrid colonies were readily isolated and propogated in continuous culture. Senescent colony morphology similar to that seen with microcell Chinese hamster X transfer was observed in cell-cell fusion studies of normal male CHE cells with a G418-resistant derivative of Ni-2/TG^R cells (*12*). In these studies, 95% of the resulting hybrids senesced in <50 days after reaching a colony size of not more than 1000 cells, indicating that microcell and cell hybrids exhibited similar patterns of clonal senescence (*12*).

To map the region of the X chromosome involved in senescence, we used other cell lines as microcell donors. Transfer of the structurally altered Xp from Ni-2 tumor cell lines (the $HPRT^+$ parent of Ni-2/TG^R) did not suppress the immortal phenotype of the Ni-2/TG^R cells (Table 1); of 16 hybrid

Table 1. Microcell fusions with the Ni-transformed lines. The recipient Ni-2/TG^R line for most of these experiments was an $HPRT^-$ variant of the Ni-transformed Ni-2 cells that contained a complete deletion of Xq. This line was routinely maintained in complete purine-free α-minimum essential medium (MEM) (Hazelton) containing 6-thioguanine (10 μg/ml). The other recipient cell was an Ni-6/TG^R cell line that contained a structurally intact X chromosome. This cell line was maintained in a similar manner as the Ni-2/TG^R. A mouse A9 line, also $HPRT^-$, was fused with normal male CHE cells. After selection in HAT medium [DMEM, with HAT (100 μM hypoxanthine, 5 μM amethopterin and 16 μM thymidine)], the cells were pooled and microcells were generated for Chinese hamster X chromosome–specific transfer to the A9 cells. The hybrid clones A9-CX 1 and 2 were isolated from this last microcell fusion by selection in HAT. Retention of an intact Chinese hamster X chromosome in these donor cell hybrids was confirmed by karyotyping. Microcells of A9-CX 1 or 2, Ni-2, or Ni-1 were fused with Ni-2/TG^R, with the exception of microcells of A-9 CX-2 (passage 9–11) which were fused with Ni-6/TG^R; fusions were according to the technique of Fournier and Ruddle, with some modifications (*14, 15*). Microcell hybrids were selected in complete α-MEM containing HAT. Ni-2/TG^R grown in HAT did not produce any colonies in more than 25 experiments. CH, Chinese hamster.

Microcell donors	Passage no. of donor cells	Transferred chromosomes	Total no. of colonies obtained	No. of colonies that senesced	Percent of senescing colonies
A9-CX clone 1	4–5	Normal CH X	21*	21	100
A9-CX clone 2	4–5	Normal CH X	25†	25	100
A9-CX clone 2	6–10	Normal CH X	30†	14	47
Ni-2 cell line	6–10	CH X(del Xq1-Xqter)	16‡	0	0
Ni-1 cell line	11	CH X(del Xq3-Xqter)	1§	0	0
A9-CX clone 2	9–11	Normal CH X	32‖	24	75

*Colonies were obtained from a total of three experiments. †Colonies were obtained from six experiments. ‡Colonies were obtained from seven experiments. §Colonies were obtained from one experiment. ‖Colonies were obtained from two experiments (recipient cells were Ni-6TG^R).

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clones obtained in seven experiments, none senesced. Senescence also did not result from transfer of an incomplete X (del Xq3 → Xqter) from the Ni-transformed Ni-1 cell line (Table 1). These experiments show that only the normal Chinese hamster X chromosome was capable of inducing senescence of the immortal Ni-2/TG^R cells and suggest that the putative senescence gene may have been on the terminal region of the heterochromatic long arm of the X chromosome.

Senescence of the Ni-2/TG^R cells was induced in 100% of the hybrids that resulted from the fusion of early passage (P.4–5) A9-CX donor cells. However, with transfer of the Chinese hamster X chromosome from later passage (P.6–10) A9-CX donor cells, only 47% of the resulting hamster-mouse hybrid clones senesced. Eleven nonsenescent hybrid clones that arose from late passage A9-CX fusions were isolated and expanded into cell lines for further study. Evidence of successful X chromosome transfer in these nonsenescent hybrids was provided by karyotypic analysis, which revealed that 8 of the 11 hybrids maintained at least one intact Chinese hamster X chromosome, as confirmed by both G- and C-banding. Two hybrids acquired two transferred X chromosomes, suggesting that acquisition of multiple copies of X was not growth inhibiting. Of the three hybrids without an intact Chinese hamster X chromosome, one (X-Ni2-12) received an intact X but subsequently lost a portion of the Xq arm after translocation of 1q onto the short arm of the X; the other two (X-Ni2-3 and X-Ni2-5) carried a large marker chromosome that was likely to contain Chinese hamster X material because they were resistant to HAT. At least five of these nonsenescing hybrids appeared to have lost the parental Ni-2/TG^R Xp. Ten of these nonsenescent hybrids were remarkably similar in their modal chromosome content (n = 22 to 25) and composition to the recipient Ni-2/TG^R cells (n = 22), and one hybrid line remained stably near tetraploid (n = 48).

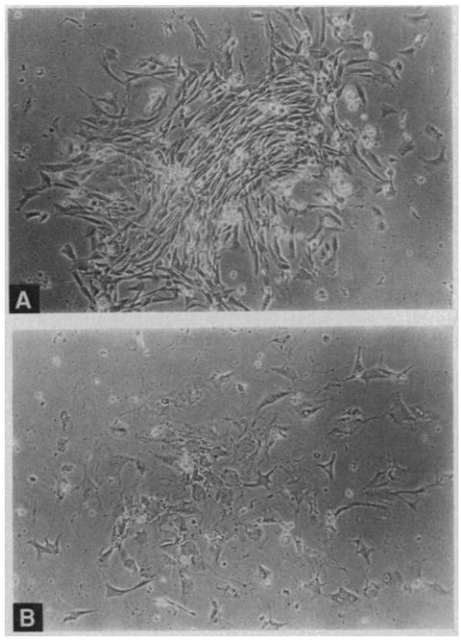

Fig. 1. The morphology of nonsenescing and senescing microcell hybrids. Cellular morphology was monitored by photography under an inverted–phase contrast microscope at various days after selection in HAT. One criterion by which hybrid clones were scored as senescing colonies was their flattened cell morphology. The difference between nonsenescing growth and senescent colonies is depicted. (**A**) A nonsenescing clone, Xp-Ni2-1, which received a transformed Xp chromosome by microcell fusion and exhibited growth characteristics identical to those of the parent cells, Ni-2/TG^R. (**B**) An example of a senescing clone (Ni-2/TG^R), which was the recipient of a normal Chinese hamster X chromosome and senesced in situ.

For most (8/11) of the nonsenescent Ni/TG^R cells with an introduced Chinese hamster X chromosome, the ability to grow in soft agar was significantly reduced as compared with the tumorigenic Ni-2/TG^R recipient cells. For Ni-2/TG^R, 30% of the cells plated formed colonies in soft agar. Of 11 Ni-2/TG^R cells with an introduced X chromosome, 8 showed a decrease in colony-forming ability to between 0 and 16% of plated cells. Although immortality and anchorage independence are both characteristic of fully tumorigenic cell lines, these data show that these cell growth parameters could be uncoupled. Tumorigenicity studies to determine the effects of the transferred intact X in nonsenescing hybrids on tumor formation in nude mice have been completed (*13*). Although many (6/11) of these hybrid cell lines yielded tumors at 100% of the injection sites (2×10^6 or 5×10^6 cells injected at four sites for each cell line), many tumors arose with an increased latency (31 to 70 days) as compared to tumor formation by Ni-2/TG^R cells (27 days). Additionally, the tumor growth rate (mm^3/day) was 40 to 80% slower than that of tumors that arose from the Ni-2/TG^R cells.

Microcell transfer of the intact Chinese hamster X from late passage A9-CX cells induced senescence of 75% of the hybrids obtained by microcell-mediated fusion of A9-CX microcells to another immortal and tumorigenic Ni-transformed cell line Ni-6/TG^R (Table 1). Ni-6/TG^R cells have a cytogenetically intact X chromosome, but may have possessed a submicroscopic deletion or other genetic alteration of this chromosome. The finding that a normal Chinese hamster X induced a high degree of senescence of the immortal Ni-6/TG^R cells suggested that the X in these immortal cells may also have lost or inactivated a senescence gene, possibly resulting from DNA damage by the transforming Ni treatment. This gene inactivation, however, did not appear to involve visible chromosomal deletion, which has been frequently associated with the loss of suppressor gene function (*3, 14*).

Table 2. Effects of azacytidine treatment of donor cells on hybrid senescence. Later passage Chinese hamster X donor (A9-CX clone 2, P. 8–10) were treated with 5-azacytidine (3 μM; Sigma, #A2385) for the time periods indicated before microcell transfer. The cells were replated into Costar flasks (25 cm^2) and allowed to attach overnight. Some cultures were given a recovery period before colcemid was added. Microcells were prepared and fused with Ni-2/TG^R as described (*10*).

Time of treatments (hours)	Recovery period (hours)	No. of colonies that senesced/total number of surviving colonies	Percent of colonies that senesced
0	0	14/30*	47
48	48	13/14†	93
72	0	4/4‡	100
72	24	9/9§	100

*Data are presented in detail in Table 1. †Data are from three experiments. ‡Data are from one experiment. §Data are from two experiments.

The intact Chinese hamster X, which was recovered in most of the nonsenescent Ni-transformed cells, may possibly have become partially inactivated during continued passage in A9 cells, since the mouse A9 cells were likely to maintain only one active X chromosome. One mechanism that can lead to gene inactivation is methylation of cytosines in certain CpG sequences (*15*). Gene inactivation can occur on an individual basis (*16*) and reactivation of a single gene on an inactive X chromosome has also been observed (*17*); thus, the entire X chromosome need not be active or inactive. Reactivation of previously methylated, inactive sequences can occur by incorporation of 5-azacytidine into DNA, since these azacytidine residues are no longer subject to methylation (*17*). To investigate whether gene inactivation had altered the activity of the Chinese hamster X chromosome during its residence in the A9 cells, we treated later passage (P.8–10) A9-CX donor cells that exhibited 50% of the senescing activity of the early passage cells (Table 1) with 5-azacytidine before microcell fusion to Ni-2/TG^R cells. Senescence again predominated among the fusion hybrids after azacytidine treatment (Table 2). These results indicated that expression of senescence genes may be controlled by epigenetic mechanisms, which are known to be heritable and may be important in oncogenesis.

In summary, the nonrandom deletion of Xq in several immortal male Ni-transformed Chinese hamster cell lines suggested that this region of the X chromosome may harbor one or more senescence-inducing sequences, since microcell transfer of an intact Chinese hamster X chromosome resulted in dominant clonal senescence. Reintroduction of an active X chromosome may have complemented a genetic defect, possibly the loss of a growth regulatory gene. The high incidence of male transformations in Chinese hamsters suggested that inactivation of this senescence gene was important in Ni transformation and further suggested that female cells may be more resistant to transformation by this deletion mechanism if this gene was active in both of the female X chromosomes. Negative growth control has been attributed to a tumor suppressor gene (*18*), and repression of c-*fos* expression has been described in senescent human fibroblasts (*19*). The X chromosome has also been reported to be active in oncogenic transformation. A human synovial sarcoma with an X;18 translocation has been reported (*20*), and the *mcf-2* and A-*raf*-1 proto-oncogenes and tissue inhibitor of metalloproteinases, a putative suppressor gene, have been localized to the human X chromosome (*21*). The highly conserved nature of X chromosomal genes (*22*) suggests that the human X chromosome may also contain a senescence-inducing gene, and in preliminary experiments we have also observed senescing activity of the human X chromosome (*23*). Loss or inactivation of this senescence gene by hypermethylation may be associated with the acquisition of immortality, and may represent an early event associated with Ni-induced transformation.

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Effect of Cytochrome P450 Arachidonate Metabolites on Ion Transport in Rabbit Kidney Loop of Henle

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In the medullary segment of the thick ascending limb of the loop of Henle (mTALH), arachidonic acid (AA) is metabolized by a cytochrome P450–dependent monooxygenase to products that affect ion transport. The linkage between changes in ion transport and AA metabolism in isolated cells of the mTALH was examined. AA produced a concentration-dependent inhibition of ^{86}Rb uptake—an effect that was prevented by selective blockade of cytochrome P450 monooxygenases. Inhibition by cytochrome P450 blockade of the effect of AA on ^{86}Rb uptake could be circumvented by addition of the principal products of AA metabolism in the mTALH.

THE MEDULLARY SEGMENT OF THE thick ascending limb of the loop of Henle (mTALH) contributes to the regulation of extracellular fluid volume as indicated by its high capacity to reabsorb NaCl, thereby establishing the solute gradient for water reabsorption (*1*). The mTALH serves as the principal site of action of the most potent class of diuretic agents, which comprises furosemide and the other "loop" diuretic drugs (*1*). In cells isolated from the mTALH of the rabbit, arachidonic acid (AA) is specifically metabolized by a cytochrome P450–dependent pathway to one or more products that can affect Na^+- and K^+-dependent adenosine triphosphatase (Na^+,K^+-ATPase) activity when tested in vitro on cell membranes (*2, 3*). A local or circulating (*4, 5*) modulator of the Na^+,K^+-ATPase in vivo has long been sought. We propose that cytochrome P450 AA metabolites influence transport function locally in the mTALH.

In order to demonstrate that ion transport in intact mTALH cells is linked to AA metabolism, we studied the effects of cytochrome P450 AA metabolites on transport by measuring ^{86}Rb uptake in cells isolated from the mTALH and manipulating the flux of AA through the cytochrome P450 pathway. Movement of ^{86}Rb reflects that of K^+ and is a reliable estimate of ion

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