REFERENCES AND NOTES

1. V. M. Ingram, *Nature* **178**, 792 (1956); *ibid.* **180**, 326 (1957).
2. J. Hofrichter, P. D. Ross, W. A. Eaton, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4864 (1974); B. C. Wishner, K. B. Ward, E. E. Lattman, W. E. Love, *J. Mol. Biol.* **98**, 179 (1975); R. H. Crepeau, G. Dykes, R. Garrell, S. J. Edelstein, *Nature* **274**, 616 (1978); G. W. Dykes, R. H. Crepeau, S. J. Edelstein, *J. Mol. Biol.* **130**, 451 (1979); E. A. Padlan and W. E. Love, *J. Biol. Chem.* **260**, 8280 (1985); W. A. Eaton and J. Hofrichter, *Blood* **70**, 1245 (1987).
3. H. F. Bunn and B. G. Forget, *Hemoglobin: Molecular, Genetic, and Clinical Aspects* (Saunders, Philadelphia, 1986).
4. R. R. Behringer *et al.*, *Science* **245**, 971 (1989).
5. Cosmids that contained HS I–V α or Hs I–V $\beta^s$ were constructed as described for HS I–V β (*9*). The human α1-globin gene is within a 3.8-kb Bgl II–Eco RI fragment and the $\beta^s$-globin gene is within a 4.1-kb Hpa I–Xba I fragment.
6. R. L. Brinster, H. Y. Chen, M. E. Trumbauer, M. K. Yagle, R. D. Palmiter, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4438 (1985).
7. L. C. Skow *et al.*, *Cell* **34**, 1043 (1983).
8. T. M. Townes, H. Y. Chen, J. B. Lingrel, R. D. Palmiter, R. L. Brinster, *Mol. Cell. Biol.* **5**, 1977 (1985); T. M. Ryan, R. R. Behringer, T. M. Townes, R. D. Palmiter, R. L. Brinster, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 37 (1989).
9. T. M. Ryan *et al.*, *Genes Dev.* **3**, 314 (1989).
10. Globin mRNA levels were quantitated by solution hybridization as described by T. M. Townes *et al.*, *EMBO J.* **4**, 1715 (1985).
11. Accurate quantitation of Hb S in mice of the "single" haplotype is difficult because of heterodimer formation (*4*) in IEF gels; the top band, $h\alpha_2h\beta^s_2$, in Fig. 2 is contaminated with $h\alpha_2m\beta_2$ and the bottom band, $m\alpha_2m\beta_2$ is contaminated with $m\alpha_2h\beta^s_2$. The ratio of $h\alpha_2h\beta^s_2$ to $m\alpha_2m\beta_2$ may actually be increased in the Hb S/β-thal mice but the change may be obscured by an increase in $m\alpha_2h\beta^s_2$ in the bottom band. The $\beta^s$ polypeptides are presumably in excess because there is five times more human $\beta^s$-globin mRNA than mouse β-globin mRNA in the Hb S/β-thal animals (see Fig. 3). However, direct measurement of $\beta^s$-globin levels is difficult because human $\beta^s$ and mouse $\beta^{single}$ polypeptides migrate at the same position on denaturing gels.
12. Cells were suspended in saline buffered with 10 m*M* sodium phosphate (*p*H 7.4) and slowly (3 to 5 ml/min) deoxygenated with nitrogen gas [K. Horiuchi *et al.*, *Blood* **71**, 46 (1988); T. Asakura and J. Mayberry, *J. Lab. Clin. Med.* **104**, 987 (1984)].
13. For scanning electron microscopy deoxygenated RBCs were fixed in 2% glutaraldehyde in cacodylate buffer, *p*H 7.2, for 5 days. The fixed cells were washed with cacodylate buffer and collected on a filter. The filter was floated in 2% osmium tetroxide for 10 min and dehydrated in a graded series of ethanol and dried with a Deuton Vacuum apparatus. The specimen was splattered with platinum palladium and examined with a Philips scanning electron microscope. For transmission electron microscopy, deoxygenated RBCs were fixed overnight in 2% glutaraldehyde in cacodylate buffer, *p*H 7.2, washed with cacodylate buffer and treated with 2% osmium tetroxide for 1 hour. The samples were dehydrated in a graded series of ethanol and embedded in Epon resin. Thin sections were cut with an LKB Ultrotome III and examined with a Philips 300 electron microscope.
14. In principle the ratio of Hb S to mouse Hb can be increased by making the mice homozygous for the transgenes and homozygous for the β-thal mutation. One advantage of this transgenic line is that different ratios of Hb S to mouse Hb can be attained by the appropriate genetic crosses. However, it is possible that progeny with higher Hb S to mouse Hb ratios will not be viable because higher Hb S levels in early development may not be tolerated.
15. The human α- and $\beta^s$-globin oligonucleotides used in the primer extension analysis were as described (*4*). The mouse βh1 primer 5′-ATAGCTGCCTTC-TCCTCAGCT-3′ corresponds to sequences from +67 to +87 of the mouse βh1 gene.
16. J. B. Whitney III, R. R. Cobb, R. A. Popp, T. W. O'Rourke, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7646 (1985).
17. We thank N. Martin, J. Askins, and M. Avarbock for excellent technical assistance and J. Barker for providing the *Hbb*$^{d\text{-}3thJ}$ β-thalassemic mice. Supported in part by grants HL-35559, HL43508, HD-09172, and HD-23657 from the National Institutes of Health. T.R. is a predoctoral trainee supported by National Institutes of Health grant T32 CA-09467.

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# Tumorigenicity in Human Melanoma Cell Lines Controlled by Introduction of Human Chromosome 6

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**Chromosome banding analysis of human malignant melanoma has documented the nonrandom alteration of chromosome 6. To determine the relevance of chromosome 6 abnormalities in melanoma, a normal chromosome 6 was directly introduced into melanoma cell lines. The resulting (+6) microcell hybrids were significantly altered in their phenotypic properties in culture and lost their ability to form tumors in nude mice. The loss of the chromosome 6 from melanoma microcell hybrids resulted in the reversion to tumorigenicity of these cells in mice. The introduction of the selectable marker (psv$_2$neo) alone into melanoma cell lines had no effect on tumorigenicity. These results support the idea that one or more genes on chromosome 6 may control the malignant expression of human melanoma.**

MALIGNANT TRANSFORMATION IS envisioned as a multistep process (*1*) with recent studies highlighting the importance of tumor suppressor genes in the development of human cancers (*2*). Although evidence supporting the existence of tumor suppressor genes (whose inactivation would release a cell from normal growth control) is accumulating (*3*), to date, only two tumor suppressor genes (*RB* and p53) have been cloned (*4*). Evidence supporting the presence of tumor suppressor genes in human malignancies has primarily come from two lines of investigation: (i) the study of allelic loss [by restriction fragment length polymorphism (RFLP) analysis] and (ii) the study of somatic cell fusion experiments. The former experimental approach has documented allelic loss of specific chromosome regions in several cancers including colon cancer, lung cancer, Wilms' tumor, retinoblastoma, and neuroblastoma (*5, 6*). In melanoma, RFLP allelic loss of several chromosome regions has been described for melanoma cell lines (*7*). In addition to reports of allelic loss, direct support for the presence of suppressor genes in cancer has resulted from the use of interspecific (*8*) and, more recently, intraspecific human somatic cell hybrids (*9*). Somatic cell hybridization involves the introduction of a complete genome from a normal cell into a cancer cell via cell fusion. In contrast, by microcell hybridization, the effect of introducing a single human chromosome into a recipient cell can be studied (*10*).

Our reason for selecting human chromosome 6 for microcell hybridization relates to its frequent involvement in structural alterations in melanoma (*11*). The most frequent alteration of chromosome 6 in melanoma is simple deletion of the long arm (6q−) (*11*), although a nonreciprocal translocation site involving chromosome 6 (leading to loss of sequences on distal 6q) has been described (*12*). Almost 40% of melanomas exhibit loss of sequences on 6q (*11, 12*). We report here the introduction of a chromosome 6 derived from a normal human diploid fibroblast into melanoma cell lines. Our results suggest that one or more genes on chromosome 6 have a role in tumorigenic expression of human melanoma.

A human-mouse microcell hybrid was generated that contained a single copy of chromosome 6 (derived from a normal human diploid fibroblast) as its only human component (*13*). Retention of chromosome 6 resulted from the insertion of the neomycin antibiotic resistance gene (psv$_2$neo) into

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this chromosome 6, and subsequent selection of microcell hybrids with medium containing the neomycin analog G418 (600 μg/ml). The chromosome 6–bearing hybrid (termed MCH-262A1D6) was then used to transfer chromosome 6 into two human melanoma cell lines (UACC-903 and UACC-091) via microcell transfer. A summary of characteristics of the parental and microcell cell lines is presented in Table 1.

With the UACC-903 microcell hybrid, the introduction of chromosome 6 caused a dramatic change in cell morphology. Parental cells grew quickly to confluence and formed foci even at low plating densities (Fig. 1A). In contrast, after the introduction of a chromosome 6, the resulting (+6) cell hybrids displayed a flattened stellate appearance and failed to produce foci even at high plating densities (Fig. 1B). Also, the cell generation time of the (+6) microcell hybrid was significantly lengthened as compared to the parental cell line (4.6 versus 2.7 days, respectively). The ability of parental and microcell clones to form colonies in soft agar was also examined. The soft agar cloning efficiency was profoundly reduced in the (+6) microcell clone (Table 1). We then examined by immunohistochemistry the reactivity of a monoclonal antibody [HMB-45 (*14*)] specific for melanocytic tumors on parental and (+6) microcell hybrids. Whereas the parental UACC-903 cell line stained very strongly for HMB-45, staining of the (+6) microcell hybrid for HMB-45 was markedly reduced (Fig. 2).

In vitro morphology and HMB-45 staining were also altered in two different (+6) microcell hybrids of the UACC-091 cell line when compared to the parental line. In vitro, cells from the (+6) microcell hybrid were significantly larger and more flattened in appearance than the parent cells. By HMB-45 staining, the parent displayed strongly positive staining, which was localized in diffuse globules in the cytoplasm. In contrast, the (+6) microcell hybrid of UACC-091 displayed only occasional staining, which invariably was perinuclear, possibly associated with the Golgi apparatus. One of the two microcell hybrids showed a reduction in soft agar clonogenicity, whereas both showed a lengthening of cell generation time (Table 1). The dramatic decline in melanoma-associated antigen expression, together with the finding of altered cell morphology and diminished agar clonogenicity of (+6) microcell hybrids, suggests that the introduction of a normal chromosome 6 into these melanoma cell lines leads to a suppression of the in vitro–transformed phenotype.

The introduction of a normal chromosome 6 into cells after microcell hybridization was documented by both cytogenetic analysis of microcell hybrids and examination of RFLP allelic addition with polymorphic DNA probes. By chromosome banding analysis (*15*), the UACC-091 parental cell line demonstrated a deletion of chromosome 6 at band q16 [del(6)(q16)], whereas the UACC-903 cell line demonstrated two apparently normal chromosome 6's (Fig. 3A). Results of chromosome banding analysis comparing the parental cell line with the (+6) microcell hybrids of both the UACC-903 and UACC-091 cell lines were consistent with the introduction of a complete

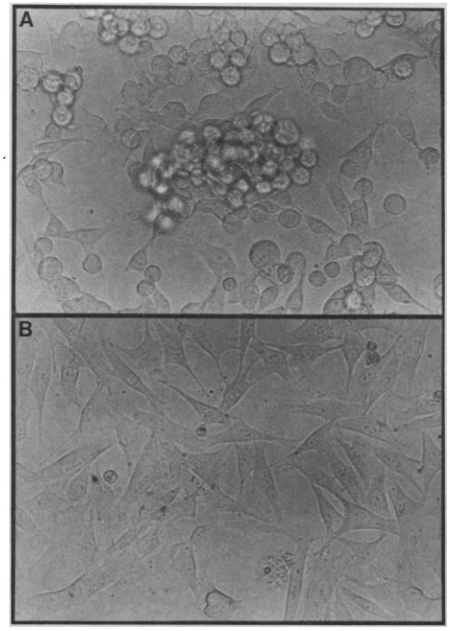


**Fig. 1.** Phase micrographs of monolayer cultures of the parental (**A**) and (+6) microcell hybrid (**B**) of the human melanoma cell line UACC-903. (A) Cells of the parental melanoma line displayed a grape-like appearance and had the propensity to form foci even at low plating densities (×100). (B) In contrast, the (+6) microcell hybrid displayed a more differentiated appearance with distinct nuclei and nucleoli and a flattened stellate morphology. Further, cells with the introduced chromosome 6 failed to form foci even at high plating densities (×100).

**Table 1.** Properties of melanoma cell lines subsequent to introduction of human chromosome 6. The parental UACC-903 and UACC-091 cell lines were obtained from the Arizona Cancer Center's Tissue Culture Core Service. The parental lines were both killed within 2 weeks with the selecting dose of G418 used to maintain the microcell hybrids (600 μg/ml). The introduction and origin of the introduced chromosome 6 was determined by a combination of cytogenetic analysis (to document the presence of an entire additional chromosome 6) and RFLP analysis (using polymorphic probes for chromosome 6). In cases where tumors arose after growth delay in vivo, the retention of the introduced chromosome 6 was tested directly on tumor cell DNA. Tumorigenicity was determined by the progressive growth after injection of $5 \times 10^6$ to $1 \times 10^7$ tumor cells per injection site in 4- to 6-week-old athymic *nu/nu* mice. Soft agar cloning efficiency was calcualted as previously described (*18*) by suspending $1 \times 10^4$ cells in 0.33% soft agar in α–minimum essential medium and counting colonies of >50 μm at 21 days. The cell generation time was calculated with the mathematical technique of Leibovitz and Mazur (*19*): $n = 3.32 (\log N - \log X_0)$, where $n$ is the number of generations, $N$ is the final population, and $X_0$ is the initial population. The monoclonal antibody HMB-45, which is specific for melanocytic tumors (*14*), was used to evaluate the immunocytochemistry of melanoma cell lines. Reactivity to HMB-45 was quantitated as uniformly positive [>80% of all tumor cells positive (+++)] to focal reactivity [<10% of tumor cells positive (−−+)].

| Cell line | Transformed phenotype (Yes/No) | Growth in G418 (+/−) | Chromsome 6 introduced | No. tumors/ no. injection sites (day 10) | Soft agar cloning efficiency (%) | Cell generation time | HMB expression (+/−) |
|---|---|---|---|---|---|---|---|
| | | | *Parental cells* | | | | |
| Parental UACC-903 | Yes | − | | 20/20 | 3.6 | 2.7 | +++* |
| Parental UACC-091 | Yes | − | | 12/12 | 5.4 | 2.0 | +++ |
| | | | *Microcell hybrid clones* | | | | |
| UACC-903 MCH-361C1 | No | + | 6 | 0/12† | 0.2 | 4.6 | −−+ |
| UACC-903 MCH-361C1*R*‡ | Yes | − | − | 6/6 | ND | ND | +++ |
| UACC-091 MCH-360C1 | No | + | 6 | 0/12§ | 5.4 | 2.7 | −−+ |
| UACC-091 MCH-360C4 | No | + | 6 | 0/12§ | 2.9 | 6.1 | −−+ |
| | | | *G418-resistant line* | | | | |
| UACC-903 + psv$_2$neo | Yes | + | | 8/8 | ND | ND | ND |

*Reactivity to HMB-45 was quantitated as uniformly positive to focal reactivity (see Fig. 2). †Tumors arose in all animals after growth delay of up to 34 days. In every case examined, loss of the introduced chromosome 6 [assessed by cytogenetic and RFLP analysis (Fig. 3B)] occurred coincident with tumor formation. ‡This revertant line was produced by culturing cells from a tumor formed after injection of MCH-361C1. §After 60 days, 40% of animals developed tumors. However, loss of the introduced chromosome 6 was again confirmed in all cases studied by directly analyzing tumor tissue by RFLP analysis.

copy of chromosome 6. These results were corroborated by RFLP analysis. Analysis of DNA from the parental UACC-903 melanoma cell line, the (+6) microcell hybrid of UACC-903, and the (+6) bearing–mouse A9 cell line used for microcell hybridization are shown in Fig. 3B. For the polymorphic probe D6S37 [which maps to the distal long arm of chromosome 6 (*16*)] restriction fragments unique to the introduced chromosome 6 were unequivocally identified in the (+6) microcell hybrid. Similar evidence for introduction of chromosome 6 into the UACC-091 cell line was also documented by RFLP analysis (*17*).

To determine the in vivo relevance of chromosome 6 to tumorigenicity, we evaluated both melanoma cell lines and their (+6) microcell hybrids for growth in athymic (*nu/nu*) mice. After injection of tumor cells ($5 \times 10^6$ to $1 \times 10^7$) from the parental cell lines (UACC-903 and UACC-091), rapid growth occurred and palpable tumors developed in 100% of animals within 5 to 7 days (Table 1).

For the UACC-903 cell line, tumor formation was initially suppressed (up to 34 days) in the (+6) microcell hybrid when compared to the parental line. However, eventually all animals developed tumors. The finding of initial suppression followed by tumor formation might be due to the instability of the introduced chromosome 6 in the microcell hybrid. This was in fact demonstrated by several approaches. First, tumors arising from animals injected with (+6) microcell hybrids were resected and placed back into culture and exposed to selective G418 medium. As would be expected if the introduced chromosome 6 (bearing the $psv_2neo$ gene) had been lost, cultures reverted to neomycin sensitivity (Table 1). Second, cytogenetic analysis of the revertant microcell hybrid was consistent with the loss of the introduced chromosome 6. Third, DNA was isolated directly from the tumors that grew in animals inoculated with (+6) microcell hybrids, and loss of the introduced chromosome 6 was confirmed (Fig. 3B). The rapid segregation of cells without the introduced chromosome 6 in our microcell hybrids may be related to the retention of the introduced chromosome 6 in a micronucleus (Fig. 3C). Whereas the parental cell line contained no cells with micronuclei, we found >30% of the (+6) microcell hybrids had a single micronucleus. When tumor cells from the (+6) microcell "revertants" were examined, far less than 1% of cells had a micronucleus. Fourth, when cells that had lost the introduced chromosome 6 were reintroduced into nude mice, they exhibited a tumor-forming capacity in mice indistinguishable from the parental cell line (that is, tumors formed in 100% of animals within 5 to 7 days). Finally, the (+6) revertant cells were analyzed and, in contrast to the (+6) microcell hybrid line, returned to the parental staining pattern for the melanoma-associated HMB-45 antibody (Table 1).

After the injection of the identical number of cells for the UACC-091 (+6) microcell hybrid cell line into mice, tumor growth was completely suppressed in all animals for 2 months. At this time, small but palpable tumors have appeared in 40% of animals. As above, RFLP and cytogenetic analysis showed that the introduced chromosome 6 had been lost from all cells reexpressing the

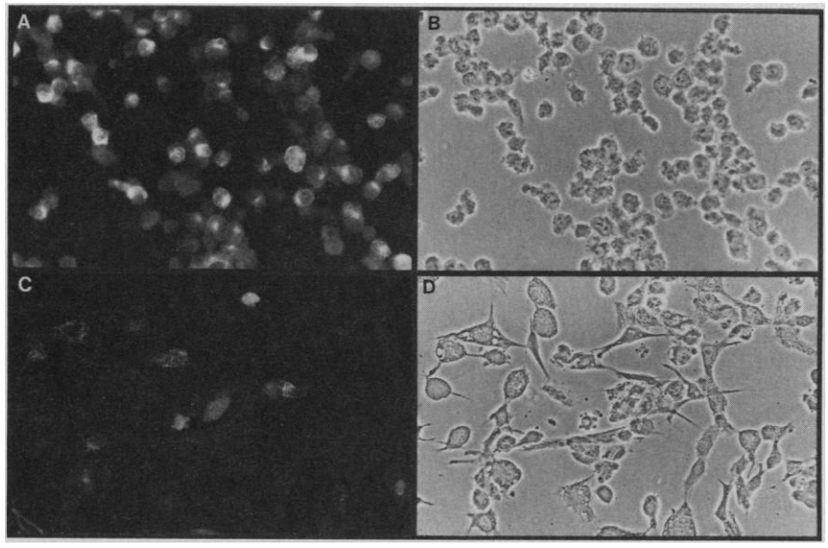


**Fig. 2.** Immunofluorescence staining of parental (**A** and **B**) and (+6) microcell hybrids (**C** and **D**) of the UACC-903 melanoma cell line. (A) Cells from the parental cell line were first grown on cover slips and then stained with an antibody highly restricted to melanoma [HMB-45 (*14*)]. The parental line stained uniformly positive for HMB-45 with almost all cells staining positively (×100). (B) A phase micrograph of the same field as (A) showing the grape-like morphology characteristic of this cell line. (C) Markedly reduced HMB-45 staining of the (+6) microcell hybrid of the UACC-903 cell line (×100). (D) Phase micrograph of the same field as (C). Note again the more flattened elongated cell morphology of the (+6) microcell hybrid.

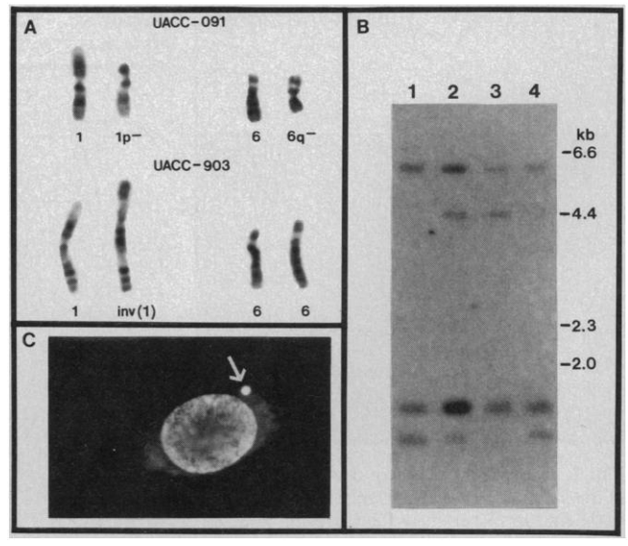


**Fig. 3.** (**A**) G-banded chromosomes illustrating clonal structural alterations from UACC-903 and UACC-091. Both lines had structural alterations of chromosome 1 [UACC-091 del(1)(p22); UACC-903 derinv(1)(p36)(q25)]. Only the UACC-091 cell line showed a recognizable structural alteration of chromosome 6 [del(6)(q16)]. Further description of cytogenetic changes characterizing these cell lines is provided in (*15*). (**B**) A Southern (DNA) blot filter containing 5 μg of Hind III–digested DNA from the UACC-903 melanoma cell line (lane 1), the (+6) microcell hybrid of UACC-903 (MCH-361C1) (lane 2), the A9 mouse/human chromosome 6–bearing hybrid MCH-262A1D6 (lane 3), and a revertant (MCH-361C1R) (lane 4) of the (+6) microcell hybrid from lane 2. DNA was hybridized with probe pJCZ30 (D6S37) in 0.15*M* NaCl, 0.03*M* tris-HCl (*p*H 7.4), 2 m*M* EDTA, 0.1% NaPPi, 0.1% SDS, 10% dextran sulfate, heparin (0.5 mg/ml), and salmon sperm DNA (100 μg/ml) at 65°C. The filter was washed for 1.5 hours in 0.1× saline sodium citrate and 0.1% SDS at 60°C. The filter was exposed overnight at −80°C. (**C**) Example of a micronuclei within a cell from the (+6) microcell hybrid of UACC-903. The cell was stained with the DNA fluorochrome Hoechst 33258 (which stains DNA brightly) and the arrow points to the micronucleus within the cytoplasm (×220).

tumorigenic phenotype. All other animals have remained tumor-free. For animals without tumors, histopathologic examination of the site of injection of the (+6) microcell hybrids after 2 months shows only occasional reactive fibroblasts with no evidence of melanoma cells. In contrast, the histology of the parental and revertant tumors grown in nude mice are indistinguishable from the patient's melanoma.

As a control for the introduction of chromosome 6 into melanoma cell lines, we have introduced, by electroporation, $psv_2neo$ into the parental UACC-903 melanoma cell line (Table 1). In contrast to the introduction of chromosome 6, the addition of the selectable marker did not alter in vitro morphology or effect tumorigenicity, suggesting it has no role in the suppression of tumor formation.

Several studies have been reported on the genetics of tumor suppression by somatic cell fusion (*8, 9*). However, only recently has intraspecies hybridization of a single human chromosome been possible (*10*). On the basis of our current observations, it is reasonable to speculate that genetic information present on chromosome 6 can suppress the malignant phenotype of human melanoma cells. If [as suggested by Vogelstein (*5*)] tumor suppressor genes have both a hierarchical and incremental effect on the regulation of cell growth, it is possible that a gene, or genes, on chromosome 6 may be acting early in the pathway of tumor formation.


REFERENCES AND NOTES

1. P. Armitage and R. Doll, *Br. J. Cancer* **8**, 1 (1954); A. Knudson, Jr., *Cancer* **35**, 1022 (1975).
2. A. Knudson, Jr., *Cancer Res.* **45**, 1437 (1985); M. Hansen and W. Cavenee, *ibid.* **47**, 5518 (1987); E. J. Stanbridge, *Adv. Virol. Oncol.* **6**, 83 (1987); R. Weinberg, *Cancer Res.* **49**, 3713 (1989).
3. E. J. Stanbridge, *Bioessays* **3**, 252 (1985); G. Klein, *Science* **238**, 1539 (1987); S. Friend, T. Druja, R. Weinberg, *N. Engl. J. Med.* **318**, 618 (1988).
4. S. Friend *et al.*, *Nature* **323**, 643 (1986); W.-H. Lee *et al.*, *Science* **235**, 1394 (1987); Y.-K. T. Fung *et al.*, *ibid.* **236**, 1657 (1987); S. Baker *et al.*, *ibid.* **244**, 217 (1989); T. Takahaski *et al.*, *ibid.* **246**, 491 (1989).
5. B. Vogelstein *et al.*, *Science* **244**, 207 (1989).
6. K. Kok *et al.*, *Nature* **330**, 578 (1987); S. Naylor, B. Johnson, J. Minna, A. Sakaguchi, *ibid.* **329**, 451 (1987); H. Brauch *et al.*, *N. Engl. J. Med.* **317**, 1109 (1987); S. Orkin, D. Goldman, S. Sallan, *Nature* **305**, 172 (1983); E. Fearon, B. Vogelstein, A. Feinberg, *ibid.* **309**, 176 (1984); A. Koufos *et al.*, *ibid.* **316**, 330 (1985); W. Cavenee *et al.*, *ibid.* **305**, 779 (1983); C.-T. Fong *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3753 (1989).
7. N. Dracopoli, A. Houghton, L. Old, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1470 (1985).
8. H. Harris *et al.*, *Nature* **223**, 363 (1969); H. Harris, *Somatic Cell Genet.* **5**, 923 (1979).
9. E. Stanbridge, *Nature* **260**, 17 (1976); E. Stanbridge and J. Wilkinson, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1466 (1978); H. Klinger, *Cytogenet. Cell Genet.* **27**, 254 (1980); E. J. Stanbridge *et al.*, *Science* **215**, 252 (1982); W. Benedict, B. Weissman, C. Mark, E. Stanbridge, *Cancer Res.* **44**, 3471 (1984).
10. C. McNeill and R. Brown, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5394 (1980); R. Fournier, *ibid.* **78**, 6349 (1981); B. E. Weissman *et al.*, *Science* **236**, 175 (1987).
11. J. M. Trent, S. B. Rosenfeld, F. L. Meyskens, *Cancer Genet. Cytogenet.* **9**, 177 (1983); R. Becher *et al.*, *Cancer Res.* **43**, 5010 (1983); S. Pathak, H. L. Drwinga, T. C. Hsu, *Cytogenet. Cell Genet.* **36**, 573 (1983); G. Balaban *et al.*, *Cancer Genet. Cytogenet.* **11**, 429 (1984); M. I. Pedersen, J. W. Bennett, N. Wang, *ibid.* **20**, 11 (1986); J. M. Cowan, R. Halaban, A. T. Lane, U. Francke, *ibid.*, p. 255; A. H. Parmiter, G. Balaban, M. Herlyn, W. H. Clark, P. C. Nowell, *Cancer Res.* **46**, 1526 (1986).
12. J. M. Trent *et al.*, *Cancer Res.* **49**, 420 (1989).
13. Microcell hybrids are constructed by treating a proliferating population of donor cells [containing a chromosome with an integrated dominant selectable marker] with colcemid (0.15 μg/ml) for 72 hours. The cells are initially blocked in mitosis, but over time the majority of cells overcome the colcemid block and enter $G_1$. Because no spindle has formed, the chromosomes recondense into multiple micronuclei with each nucleus containing one to several chromosomes. The population of multinucleate cells is enucleated with a combination of cytochalasin B (2 μg/ml) and centrifugal force. The resulting microcells are filtered to produce a population that consists predominantly of micronuclei containing single chromosomes. These microcells are then fused to recipient cells and grown in medium containing 600 μg of G418 per milliliter. Only recipient cells into which the chromosome containing the integrated *neo* has been introduced are capable of surviving in this selective medium.
14. Cells grown on cover slips were fixed 5 min in cold methanol (4°C) and permeabilized by dipping in acetone. The primary antibody, HMB-45 [A. M. Gown *et al.*, *Am. J. Pathol.* **123**, 195 (1986)] was incubated for 30 min. Slides were then washed three times with phosphate-buffered saline (PBS). Secondary fluoresceinated rabbit antibody to mouse antigen was incubated for 30 min. Again the slides were washed three times with PBS and observed under a cover slip with an epifluorescent microscope. As described by Gown *et al.*, HMB-45 recognizes 97% of malignant melanomas (both pigmented and nonpigmented) while failing to react with normal skin melanocytes. The antibody recognizes an unknown cytoplasmic antigen. In direct screening of tumor tissues, the HMB-45 antibody in general strains "epitheloid" melanoma cells more uniformly positive than more "sarcomatous" melanoma cells (analogous to the situation following the introduction of chromosome 6 into our cell lines).
15. Exponentially growing cells were harvested for karyotypic analysis as described previously [J. Trent and F. Thompson, in *Methods in Enzymology*, M. Gottesman, Ed. (Academic Press, New York, 1987), pp. 267–278]. A minimum of 50 cells per line were analyzed, with results expressed according to International System for Human Cytogenetic Nomenclature (ISCN) recommendations [*Cytogenet. Cell Genet.* **21**, 1 (1985)]. The model chromosome number of the UACC-903 cell line was 47, including clonal structural alterations of chromosome 1 [t(1;11)(q23;q23) and derinv(1) (p36q25)]; chromosome 11 [t(1;11) and del(11) (q23)]; and chromosome 17 [t(17;?)(q25;?)]. No evidence of chromosome 6 alterations were observed in any cell (Fig. 3A). The UACC-091 cell line had a hypertriploid modal chromosome number (78 chromosomes per cell) with clonal structural alterations of chromosome 1 [del(1)(p22)]; chromosome 3 [del(3)(q13)]; chromosome 5 [t(5;?)(p14;?)]; chromosome 6 [del(6)(q16)]; chromosome 7 [iso(7q)]; chromosome 12 [del(12)(p12)]; chromosome 13 [t(13:?)(p11;?)]; chromosome 15 [t(15;?)(p11;?)]; and chromosome 16 [t(16;?)(q25;?)]. Fig. 3A documents chromosome 1 and 6 alterations from UACC-091 and chromosome 1 alterations from the UACC-903 cell line.
16. Y. Nakamura *et al.*, *Nucleic Acids Res.* **16**, 4743 (1988).
17. J. Trent, unpublished data.
18. H. McBride, B. Stea, A. Hill, J. Trent, *Int. J. Radiat. Oncol. Biol. Phys.*, in press.
19. A. Leibovitz and K. Mazur, *Tissue Cult. Assoc. Man.* **3**, 655 (1977).


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# 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: A Neuroprotectant for Cerebral Ischemia


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**2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) is an analog of the quinoxalinedione antagonists to the non–*N*-methyl-D-aspartate (non-NMDA) glutamate receptor. NBQX is a potent and selective inhibitor of binding to the quisqualate subtype of the glutamate receptor, with no activity at the NMDA and glycine sites. NBQX protects against global ischemia, even when administered 2 hours after an ischemic challenge.**


THE DISCOVERY OF THE QUINOXALINEDIONES (*1*), a series of potent and selective antagonists at non-NMDA excitatory amino acid (EAA) receptors, has greatly facilitated the study of the pharmacology of the quisqualate and kainate receptor subtypes. Here we report a new non-NMDA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) (Fig. 1), which is more potent than the previously described compounds DNQX (6,7-dinitroquinoxaline-2,3-dione) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) in displacing [$^3$H]AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) binding and is less potent in inhibiting [$^3$H]kainate, [$^3$H]CPP [3(2-carboxypiperazine-4-yl)propyl-1-phosphonic


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