

TUMOR HYPERSENSITIVE DNA IS ENRICHED IN C-MYC SEQUENCES AND
REACTS DIFFERENTIALLY WITH NORMAL AND MALIGNANT
GENOMIC DNA

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We now show that exposure of B16 melanoma cells to bromo-deoxyuridine increases cell-substratum interactions concurrent with an increase in genome susceptibility to nucleases. Hypersensitive DNA was isolated after mild nicking of nuclei with DNase I followed by repair with DNA polymerase I in the presence of biotin-19-SS-dUTP and affinity chromatography on streptavidin-agarose. Dot blot studies showed that the hypersensitive DNA is enriched in c-myc sequences compared to total tumor genomic DNA, and hybridizes preferentially to the latter, compared to normal genomic DNA, particularly when prepared from BrdU-treated cells. Since hypersensitive DNA can hybridize with multiple Alu sequences in the genome, we postulate that one of the mechanisms for its differential reactivity may be by recognition of an unequal number of Alu repeats in normal and tumor genomic DNA. © 1990 Academic Press, Inc.

Regulation of gene activity is thought to be partly mediated by changes in chromatin structure, since active genes are thought to be part of DNase I-hypersensitive sites, in contrast with the greater nuclease resistance of inactive genes (1). On the other hand, the decrease in heterozygosity for normal alleles in tumors (2) the loss or reorganization of genes during senescence (3), and the amplification and rearrangement of oncogene sequences (4) can be part of the chromosomal changes associated with growth control (5). We have now examined whether nuclease-susceptible genomic sites are differentially accessible in tumor cells with unequal degree of DNA modification and anchorage to substratum, and whether their hypersensitive DNA

has a potential in identifying sequences unequally represented in total normal or mouse genomic DNA. To evaluate this, we have now compared the genome exposure and hybridization properties of hypersensitive DNA from control B16 melanoma and the corresponding cultures in which cell adhesion is increased by growth with the DNA modifying agent, bromodeoxyuridine (BrdU), which is known to influence the malignant phenotype (7) and metastatic behaviour (8) of B16 melanoma cells. We have also investigated whether the affinity purified hypersensitive DNA prepared from control and BrdU-treated cells is enriched in growth-associated c-myc sequences (4,5) and whether it can recognize differentially tumor and normal genomic DNA.

MATERIALS AND METHODS

Metastatic B16 melanoma. BL6 cells were cultured *in vitro* in Dulbecco's medium supplemented with 4.5 g/l glucose, non-essential aminoacids and 10 % fetal calf serum, including 2.5 $\mu\text{g/ml}$ of BrdU whenever indicated for a 72 hour exposure (6).

DNase I-mediated assay or genomic susceptibility and isolation of hypersensitive DNA. Cells were exposed to a hypotonic buffer consisting of 20 mM Tris pH 7.4, 10 mM NaCl, 3 mM $\text{Mg}(\text{OAc})_2$ and 10 mM vanadyl ribonucleoside complex, for 5 min at 4°. Cell lysis was achieved by addition of Nonidet P40 to 1.2 % in the presence of 5 % sucrose. Nuclei were harvested at 1200 xg for 5 min and 500 μl aliquots containing about 1×10^9 nuclei were washed and stored in 1X buffer containing 20 mM Tris-HCl pH 7.9, 20 % glycerol, 140 mM KCl, 10 mM MgCl_2 and 1 mM DTT. DNase I treatment of nuclei was carried out with 0.1 $\mu\text{g/ml}$ of the enzyme in 50 mM Tris-HCl pH 7.5, 10 mM MgCl_2 and 50 $\mu\text{g/ml}$ of acetylated bovine serum albumin. This was followed by a 2X wash in the latter buffer and nick translation in 100 μl of the same buffer containing 20 μM of dATP, dGTP, dCTP, 10 μM biotin-19-SS-dUTP (Clontech) and 50 $\mu\text{C/ml}$ of α - ^{32}P -dTTP (3000 c/mmol; NEN) plus 20 units of DNA Polymerase I for 15 min at 15°, interval at which the reaction kinetics showed close to maximal incorporation (9). Subsequently DNA was purified by phenol extraction digested with Eco R1 and separated from bulk genomic DNA by affinity chromatography on streptavidin-agarose (Gibco-BRL), which was washed with TEN buffer (10 mM Tris-1mM EDTA pH 7.5 containing 0.2 M NaCl) to remove non-specific binding followed by specific elution of the DNA in the same TEN buffer containing 50 mM dithiothreitol at pH 8.2 (9).

Dot hybridization analysis and Southern blots. The concentration of DNA was determined by an agarose plate microassay containing the H33258 fluorescent dye (10). For dot

studies, samples were applied to Genescreen membranes held on Hybridot devices (BRL) in 10 X SSC, prepared for hybridization and autoradiography, according to Sambrook et al. (11). Southern blots (12) were preceded by restriction enzyme cutting with Hae III, Eco R₁ or Alu I (Promega) for subsequent electrophoresis on 1 % agarose gels, capillary transfer to Genescreen, hybridization and X-ray autoradiographic exposure, which were carried by standard procedures (11). Whenever indicated, specific hybridization was done with the c-myc probe ATCC 41010 and with a γ actin probe kindly provided by Dr. K. Brew. Both probes were excised from their vectors with Eco R₁ and Hind III and the corresponding inserts were labelled by nick translation with α -³²P-dCTP (11).

RESULTS

Cell flattening induced by BrdU is associated with an increased genomic exposure. In agreement with our prior findings (6,8) growth of B16 melanoma cultures with 2.5 μ g/ml of BrdU was found to increase cell attachment to substratum, Fig. 1 A,B, an effect which was associated with a greater degradation of the corresponding biotinylated hypersensitive DNA compared to that obtained from control nuclei after Eco R₁ treatment (Fig. 1, right lanes). In contrast, no such an effect of BrdU was

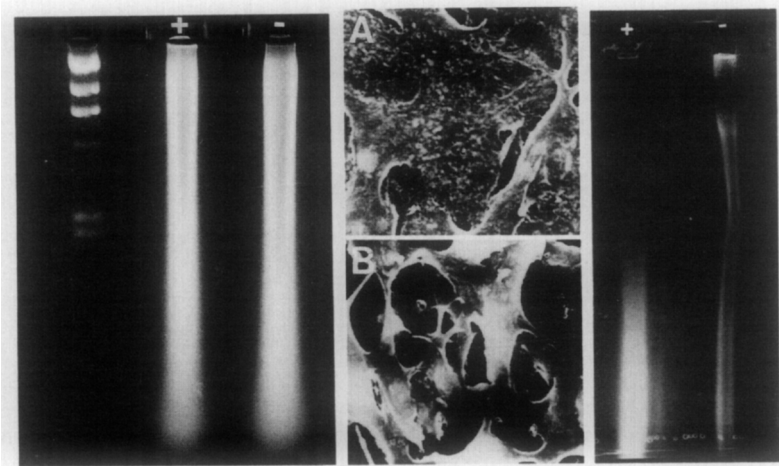


Fig. 1. BrdU-mediated cell flattening is associated with greater genomic exposure

Differential nuclease hypersensitivity of DNA in nuclei from BrdU-treated (A,+) and control (B,-) cells is shown to the right for comparison with genomic DNA obtained without nuclease pretreatment. First line to the left is a MW marker of λ DNA digested with Hind III.

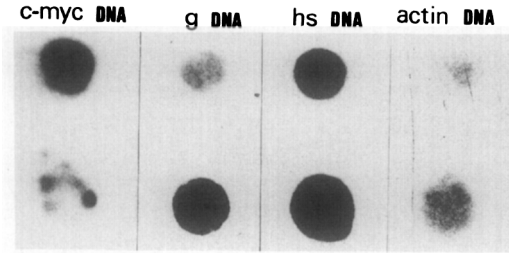


Fig. 2. Hypersensitive DNA is enriched in C-myc sequences

Dot blots from 0.125 µg of hypersensitive DNA (upper row) and 1 µg of melanoma genomic DNA were denatured and immobilized on Genescreen for hybridization with ^{32}P -labelled probes. From left, reactions obtained with: C-myc, genomic, hypersensitive and actin DNA.

evident when total genomic DNA was prepared from nuclei and treated with Eco R₁ without prior DNase I treatment and nick translation (Fig. 1, left lanes) suggesting that the effects of BrdU on melanoma cells are associated with a greater genome susceptibility to DNase I in isolated nuclei. Tumor Hypersensitive DNA is enriched in c-myc sequences and shows decreased recognition of normal genomic DNA. To determine whether the melanoma biotinylated hypersensitive DNA was enriched in c-myc specific sequences, it was physically separated from the bulk of tumor genomic DNA by chromatography on streptavidin-agarose (19) and used for dot hybridization studies. Experiments with limiting concentrations of hypersensitive DNA from BrdU-grown cells and an 8 fold greater concentration of bulk tumor genomic DNA, showed greater reactivity with hypersensitive DNA only when hybridized to c-myc DNA and significant reactivity with itself, in contrast to the greater reactivity of genomic DNA to actin DNA and to repeated sequences in itself and in the hypersensitive DNA (Fig. 2). Since BrdU-grown cells showed greater genome susceptibility to DNase I (Fig. 1) and then hypersensitive DNA could show differential reactivity with genomic DNA compared to that of control cells, we tested this possibility in dot blots.

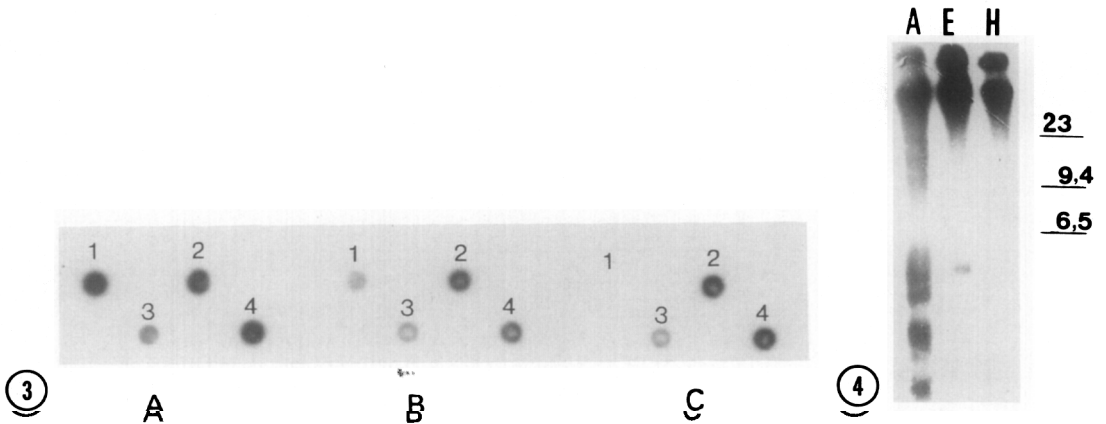


Fig. 3. Differential hybridization of Hypersensitive DNA with Normal and Tumor sequences

Replicate samples of about 0.5 μ g of DNA from normal spleen (1) melanoma (2), hypersensitive DNA from control (3) and BrdU-treated melanoma (4), were denatured and immobilized on Gene-screens for hybridization with 32 P-labelled probes from A, spleen DNA; B and C hypersensitive DNA from control and BrdU-treated cells, respectively.

Fig. 4. Multiple Alu sequences are recognized by Hypersensitive DNA

Melanoma genomic DNA was purified and digested with Hind III (H), Eco R₁ (E) or Alu I (A) for electrophoresis in 1% agarose followed by Southern blot and hybridization with hypersensitive DNA from BrdU-treated cells in the presence of competitor unlabelled spleen DNA.

For this, we used closer concentrations of normal liver genomic DNA, melanoma genomic DNA and hypersensitive DNA_s from control and BrdU-treated cells, respectively. Hybridization with normal genomic DNA showed definite signals in all four samples. However, use of tumor hypersensitive DNA as a probe showed decreased reactivity with normal genomic DNA than with tumor genomic DNA, effect which was more clearly evident with the hypersensitive DNA from BrdU-grown melanoma cells (Fig. 3).

Tumor Hypersensitive DNA recognizes multiple Alu-repeated sequences in genomic DNA. The strong hybridization of the hypersensitive DNA probe not only with itself but with the bulk of tumor genomic DNA (Fig. 2,3) suggested its reactivity with repetitive sequences. Since Alu repeats are significantly represented in the mouse genome (13), we digested

tumor genomic DNA with Alu I, Eco R₁ and Hind III for Southern blotting and molecular hybridization with the hypersensitive DNA from BrdU-grown cells, using cold sonicated mouse DNA as competition to decrease smearing of the signals with highly repeated sequences (14). This showed that Hind III cutting of the DNA did not give specific bands within the 23-0.2 kb range, but Eco R₁ gave a single component around 5 kb. In contrast, Alu I digestion of the melanoma genomic DNA showed multiple bands with unequal intensities, suggesting that the sequences present in hypersensitive DNA may be interspersed with Alu repeats (Fig. 4), which may be unequally represented in normal and tumor genomic DNA (Fig. 3).

DISCUSSION

We have now demonstrated that DNA modifying drugs like BrdU which have definite effects on tumor cell flattening can increase genome exposure in nuclei. This novel effect of the halogenated pyrimidine is in agreement with the effects of cyclic AMP on Chinese Hamster cells in which greater cell spreading is also associated with increased genome susceptibility to DNase I (15). In addition, we have now adapted a procedure for the affinity isolation of transcriptionally active murine erythroleukemia cell DNA (9) to separate melanoma hypersensitive DNA from the bulk of genomic DNA extracted from this tumor. Hybridization studies revealed a selective increase in c-myc sequences in tumor hypersensitive DNA compared to bulk tumor DNA. This differs from prior findings of c-myc amplification in total tumor DNA compared to normal DNA, and may be important since c-myc is associated with proliferation and neoplastic growth (4,5). Another novel findings was the unequal hybridization of hy-

persensitive DNA with normal and tumor genomic DNA, which was more clearly evident when extracted from BrdU-grown cells. Although the reason for this differential recognition shown by the latter hypersensitive DNA is not clear, it may be partly due to the greater nuclease susceptibility of this hypersensitive DNA which could be associated with the loss of "normal" or common repeated sequences during isolation of this DNA species or during treatment of the cells with BrdU. Nevertheless, even with hypersensitive DNA from control melanoma, we also detected greater reactivity with melanoma DNA than with normal genomic DNA from spleen or liver cells. A possible implication of our findings is that tumor hypersensitive DNA may be increased in sequences devoid of suppressor genes (16, 17), with a concurrent increase in c-myc sequences associated with cell growth.

It is known that microinjection of mRNA from senescent normal cells inhibits DNA synthesis in proliferation-competent cells (18). Moreover genomic DNA isolated from quiescent rather than from proliferating cells is known to inhibit DNA replication when transfected into tumor cells (19). Taken together, these studies suggest that growth control is partly mediated by genes whose function is to prevent proliferation. We believe that malignancy may be associated with a decreased concentration or inactivation of some of these growth-regulating genes, as has been shown for senescent cells in which some chromosomal genes decrease in concentration or acquire extra-chromosomal localization (3, 18).

Our data also suggest that Alu multiple repeats may be part of the hypersensitive DNA in both normal and tumor genomic DNA, and that variable number of these repeats may give rise to polymorphism useful in distinguishing normal and tumor

genomic DNA (14,20). In summary, our studies suggest a potential for tumor hypersensitive DNA sequences in the analysis of growth control and eventually, in tumor diagnosis.

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