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Detection of 5-Bromodeoxyuridine Incorporation in Metaphase Chromosomes of Dedifferentiated Melanoma Cells

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THESIS DEFENSE

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ABSTRACT

Observed reversible effects, mediated by the incorporation of 5-bromodeoxyuridine (BrdUrd) into the DNA of mouse melanoma cells were; changes in culture growth pattern, altered cell morphology and suppression of melanogenecis. The incorporation of BrdUrd into the metaphase chromosomes, was traced with 1.) the Hoechst 33258 fluorescence stain and 2.) the unlabeled antibody peroxidase method. Different levels of BrdUrd incorporation are shown to exhibit differential staining patterns on the metaphase chromosomes. The specific sites of preferential BrdUrd incorporation may provide a morphological clue to the structural and functional changes observed.

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INTRODUCTION

The pyrimidine, 5-bromodeoxyuridine (BrdUrd) is a base analog of thymidine, in which the methyl group is replaced by a bromine atom at the number 5 carbon. The BrdUrd competes with thymidine for incorporation sites on newly synthesized DNA, and exerts various effects on protein synthesis. Five-bromodeoxyuridine (BrdUrd), has been used by a number of investigators to study the suppression of specific cytodifferentiation in embryonic tissues involving essentially normal division of the cells. Where tested, the suppression has proven to be reversible. Coleman et al., 1970, showed that BrdUrd induced suppression in vitro of normal development of crondocytes, chick-embryo-skeletal muscle and pigmented retinal cells. Inhibition of DNA synthesis by cytosine-arabinoside C or hydroxyurea prevents BrdUrd induced suppression of normal development. Turkington et al., 1971, showed that the addition of BrdUrd to mammary epithelial cells in culture markedly inhibited milk protein induction by prolactin, after a single cell cycle. It was found that the polysome levels were depressed by 50-70%, suggesting that mRNA was affected in some way during transcription. Stellwagen and Tompkin, 1971, noted that when an established cell line of rat hepatoma was grown in the presence of BrdUrd a reversible time-dependent decrease in the activity levels of the glucocorticoid inducible liver enzyme tyrosine amino transferase, (TAT) occured. Their studies showed that BrdUrd influenced the decrease in TAT levels by lowering the rate of TAT synthesis. They concluded that BrdUrd might exert its effect at the level of the structural gene, by lowering mRNA synthesis. Weintraub

et al., 1972, has shown that a maximum of 2 cell cycles in the presence of BrdUrd is sufficient to prevent the initiation of hemoglobin synthesis in erythroblasts.

Amino acid pool differences were found between highly tumorigenic mouse melanoma cells and those which were BrdUrd-suppressed (Schuman et al., 1974). The changes in phenotype of the BrdUrd grown cells was accomplished by significant increases in the intracellular free amino acid concentration/unit of cell protein. The molar percentage of five amino acids in proteins of BrdUrd treated cells differed significantly from that of untreated melanoma cells. These observations suggest that altered amino acid metabolism is a feature characteristic of malignant cells (Hare, 1967).

Morphological, biochemical and functional observations related to the effects of exposing mouse melanoma cells to 5-bromodeoxyuridine as reported by Silagi, 1976, reveal that the degree of morphological changes, suppression of both tyrosine activity and tumorigenicity is proportional to the amount of bromouricil incorporated into the DNA. In the Silagi mouse melanoma, cell line clone $B_5$59, a highly melanotic and tumorigenic line, specific incorporation quantities of bromouricil substitution for thymine have been determined by measuring ^{3}H-BrdUrd incorporation of extracted DNA by liquid scintillation spectroscopy and X-ray fluorescence spectroscopy (Wrathall et al., 1975). When exposed to 3ug/ml of BrdUrd for 24 hours (one cell cycle) substitution of thymine by bromouricil averaged 23% and tumorigenicity was suppressed by 45%. After a 48 hour exposure, 39% substitution was recorded and an 85% suppression of tumori-

genicity. With a 72 hour exposure, 45% substitution was observed together with a 97.5% suppression of tumorigenicity (Silagi, 1976).

Ultra-structural and cytochemical studies showed an early effect in the localization of tyrosine reaction products (Wrathall et al., 1973). After 3 days of growth in BrdUrd, the melanin was no longer present in Golgi saccules and Golgi-associated smooth surface tubules, but was still seen within pre-melanosomes, compound melanosomes, and occasional Golgi-associated vesicles. Cells grown for 7 days in BrdUrd exhibit no tryosinase activity and few pre-melanosomes, which become concentrated in the juxta-nuclear region of the cell. Therefore, mouse melanoma cells are essentially amelanotic after 7 days of growth in BrdUrd. Further, after 48 hours and 72 hours, a distinct morphological change in the cells was observed, involving a flattening of the cells from a previous reticulated piled growth pattern (Silagi et al., 1971).

Tumorigenicity of mouse melanoma cells has been shown to be completely suppressed after 14 days of growth in 3ug/ml of BrdUrd (Silagi, 1972). All changes are reversible and apparently dependent on DNA synthesis. Mice injected with a clonally derived strain grown continuously in the presence of lug/ml of BrdUrd developed a degree of immunity to subsequent challenge with the 100% malignant melanoma cells in direct proportion to the number of prior injections with the BrdUrd-treated cells. Four pre-injections of BrdUrd-treated cells into susceptible animals immunized 90% of the mice (Silagi et al., 1974).

BrdUrd incorporation effects suppression of melanogenesis

of normal melanocyte cultures (Coleman et al., 1970; Zimmerman et al., 1974) and also effects a total suppression of tumorigenic potential of amelanotic melanoma cells (Silagi, 1971), other cultured tumors (Dethlefson and Tomkins, 1972) and virally transformed cells, such as polyoma-transformed mouse cells (Grady and Campbell, 1974) and SV40 transformed hamster cells (Rothchild and Black, 1973). It therefore appears that there is no obligatory interdependency or interaction between suppression of melanogenesis, tumorigenicity and altered morphological growth patterns, but possibly a common BrdUrd sensitivity or related mechanisms.

All available evidence indicates that BrdUrd exerts its affect as a result of incorporation into DNA as an analog of thymidine. When DNA synthesis is blocked or decreased by cytosine arabinoside or serum depletion during the halogenated pyrimidine treatment, no alterations in melanogenesis, tumorigenicity or morphological growth patterns occur. However, in almost every case, these effects are reversible upon subsequent cultivation in BrdUrd-free medium.

If the BrdUrd does exert its affects on the cell by incorporation into the nuclear DNA, then it should be possible to identify sites of BrdUrd incorporation on the metaphase chromosomes of such cells. Further, if the relative amounts of BrdUrd incorporation, has a regulating affect with regards to cell function, then by controlling the amount of incorporation it may also be possible to determine whether certain incorporation patterns are indicative of a cells present state. This paper reports the results of experiments designed to identify sites

of BrdUrd incorporation in the metahase chromosomes of mouse melanoma cells. An attempt is also made to compare the relative amounts of BrdUrd incorporation, and varying cell behavior, by studying incorporation patterns of BrdUrd in the metaphase chromosomes of such cells.

STATEMENT OF PROBLEM

The problem undertaken was to detect the incorporation of 5-bromodeoxyuridine in the metaphase chromosomes of mouse melanoma cells grown in tissue culture. The two cell lines that were studied are: 1.) the $B_5$59 melanoma cell line, a mouse melanoma which originated spontaneously in $C_5$7BL/6 mouse; 2.) and subclone $C_3$471, a subclone from the original $B_5$59 cell line, which has been cultured continuously in the presence of BrdUrd, at a concentration of lug/ml for several years. The $B_5$59 cells are highly melanotic and tumorigenic. However, when these cells are exposed to BrdUrd at a concentration of 3ug/ml, suppression of tumorigenicity and tyrosinase activity after 1, 2 and 3 cell cycles can be induced. The subclone $C_3$471 cells, after continuous exposure to lug/ml of BrdUrd for several years, exhibits a complete loss of tumorigenic and melanogenic capacity, but are reversible to the parental $B_5$59 cell line upon subsequent cultivation in BrdUrd-free media.

The metapahse chromosomes of the $B_5$59 cells were examined after 48 and 72 hours of exposure to 3ug/ml of BrdUrd, to determine sites of BrdUrd incorporation. The metaphase chromosomes of the subclone $C_3$471 were also examined for incorporation sites of BrdUrd. Therefore, these experiments were proposed in order to determine if any correlation may be drawn between specific sites of BrdUrd incorporation and the suppression of melanogenesis and tumorigenic potential of the $B_5$59 melanoma cells.

MATERIALS AND METHODS

Cell Cultures

The $B_5$59 cells were cultured in Eagle's minimum essential medium, supplimented with 10% fetal calf serum, 100ug/ml penicillin and streptomycin, and 265ug/ml chlortetracycline hydrochloride, Grand Island Biological Company (GIBCO). To maintain pigmented cultures, selective cloning of highly pigmented clusters was carried out at all times. A Pasteur pipette, flame polished and bent in a flame was used for aspirating, to individually harvest darkly pigmented clones of cells, and transfer them to fresh culture flasks. Falcon tissue culture flasks were seeded about 20 hours prior to the start of an experiment so that the cells would be in the logarithmic growth phase during exposure to BrdUrd. A 20 ml suspension of cells at a concentration of 2 x 10^5 cells/ml was transferred to the culture flasks and incubated at 37°C (Thelco Incubator Model 6m). After 20 hours of incubation, BrdUrd was added to each flask at a final concentration of 3ug/ml (10^{-5}M), (Nutritional Biochemical Company). The cell cultures were then incubated at 37°C, and then harvested after 48 hours at 72 hours of incubation.

The $C_3$471 cell line was cultured in the same medium as the original $B_5$59 cells, with the continuous addition of BrdUrd at a final concentration of 1ug/ml. The $C_3$471 cells were continuously cultured in the presence of BrdUrd.

Chromosome Preparations

After the desired incubation period, colcemid in Hank's Balanced Salt Solution (GIBCO) was added to each flask at a final concentration of 0.5mg/ml. The cells were allowed to in-

cubate for 2-6 hours at 37°C, the cell medium decanted, and a solution of 0.25% trypsin was added to each flask (GIBCO). The trypsin solution was allowed to remain on the cells for 1 minute and then decanted. The cell monolayers, covered by a thin film of trypsin, were then incubated at 37°C for 10 minutes. The metaphase arrested cells were collected by gentle aspiration with 10 ml of fresh medium. The cell suspension was then centrifuged at setting 3 on an International Clinical Centrifuge (Model CL), for 5 minutes. The medium was decanted and 4 ml of Phosphate Buffered Saline (PBS), pH 7.4 at 37°C was added to the cell pellet. The pellet was centrifuged once again, at setting 2 for 2 minutes, the PBS decanted, and 0.5 ml of fresh PBS was added. The cell pellet was resuspended in the PBS, being careful not to create any air bubbles. While holding the centrifuge tube at approximately a 30° angle, 4.5 ml of a 0.56% solution of sodium citrate, pH 7.6 was layered slowly on the cell suspension. The cells suspended in the PBS were then drawn up into a Pasteur pipette and slowly dropped into the sodium citrate layer. The cell suspension was then incubated at 37°C for 15 minutes to initiate swelling of the cells. The cell suspension was then centrifuged at setting 2 for 5 minutes, after which the hypotonic solution was decanted. The cell pellet was resuspended in 3 ml of Carnoy's fixative (methanol: glacial acetic acid, 3:1) and incubated at room temperature for 15 minutes. The cell suspension was centrifuged once again at setting 2 for 5 minutes. The pellet was resuspended in 3 ml of fresh Carnoy's and then incubated in a refrigerator for 30 minutes. The cell suspension was centrifuged at setting 2 for 5

minutes and resuspended in 3 ml of fresh cold Carnoy's. The cells were then dropped on glass slides. Pre-cleaned glass slides, pre-soaked in distilled water in a refrigerator, were held at a 45° angle while 2-3 drops of the cell suspension were dropped on each slide. The slides were immediately blown on to facilitate spreading of the chromosomes. The slides were allowed to air dry for 2-4 days before staining.

33258 Hoechst Fluorescent Staining

The 33258 Hoechst fluorescent dye was kindly supplied by Dr. H. Lowe of Hoechst A.G., Frankfurt am Mein, Germany. Prescreened slides with suitable metaphase chromosome spreads were stained for 15 minutes in 0.5ug/ml concentration of 33258 Hoechst in 0.14M NaCl-0.004M KCl-0.01M sodium phosphate at pH 7.0. The slides were washed for 10 minutes in the same buffer and mounted in pH 7.0 McIlvaine's buffer (0.15M sodium phosphate and 0.04M sodium citrate) with a cover slip, and ringed with Stafords rubber cement, as described by Latt et al., 1975.

The Unlabeled Antibody Enzyme Method

Immunocytochemical staining with the unlabeled antibody enzyme method using the peroxidase antiperoxidase complex (PAP) was made possible with specific anti-5-iodouracil antibodies, prepared by immunizing rabbits with bovine serum albumen conjugates of 5-iodouracil. These antibodies cross react with BrdUrd but not with thymidine (Sawicki et al., 1971). The purified serum was supplied by Drs. B.F. Erlanger and O.J. Miller, College of Physicians and Surgeons, Columbia Univeristy.

Denaturation of the DNA must precede the immunological labelling, since the antibodies react only with single stranded

DNA and freely exposed BrdUrd residues. This was carried out by placing slides into Tris buffer pH 7.6 (Sigma) and exposing them to UV irradiation from a G.E. 30 Watt germicidal lamp at a distance of 32 cm for 18 hours.

The PAP complex for use in the unlabeled antibody enzyme method was generously supplied by Dr. L.A. Sternberger, Edgewood Arsenal and Johns Hopkins University School of Medicine. Slides were pre-treated for 30 minutes with 3% normal goat serum in Tris buffer pH 7.6 (Sigma) and blotted dry. Next the primary anti-serum containing the anti-BrdUrd reacting antibodies diluted 1:30 in the buffer and supplemented with 1% normal goat serum was applied for a 30 minute incubation in a moist chamber at room temperature, followed by jet-washing with 50 ml of the buffer. The middle or bridge component of the complex, the goat-anti-rabbit serum, diluted in the buffer 1:10 was incubated and jet-washed as above. Finally, the peroxidase-anti-[redacted]xidase complex (PAP) diluted 1:60 was layered over the s[redacted]s, incubated and jet-washed. The enzyme reaction product was obtained by immersing the slides into a 0.05% solution of 3,3-diaminobenzidine tetrahydochloride (Polysciences Inc.) together with the substrate, a 0.01% Hydrogen peroxide (Fisher) solution for a reaction time of 10 minutes. After rinsing, dehydration in successively increasing concentrations of alcohol was followed with immersion in Xylene and mounting with permount (50%, 70%, 95%, 100%, 100%, Xylene for 30 seconds each).

Photography was carried out with a Leitz Dialux II fluorescence microscope with a system camera, equipped with a 200 Watt mercury lamp light source, a BG 38 heat filter, a Kp 490 di-

chroic mirror and a K510 suppression filter, For fluorescence photography, Kodak 2495 RAR film (Estar-AH Base) was used, and Kodak Plus-X film was used for photography of the immunoperoxidase labeling.

The Effect of BrdUrd Incorporation on Fluorescence of 33258 Hoechst Staining

According to the semiconservative replication of DNA, in the first generation, each daughter chromosome is composed of half old and half new DNA. The second generation, or F_2, comprises two hybrid DNA's (half old, half new) and two new DNA's made up of entirely new strands. The F_3 generation comprises two hybrid DNA's and six entirely new DNA's. When proliferating cells are exposed to a base analog such as BrdUrd, only newly synthesized strands of DNA will contain the BrdUrd. Figure 1 illustrates the expected BrdUrd substitution per DNA strand of a single chromosome after 1, 2 and 3 subsequent replications in the presence of BrdUrd. Clone $B_5$59 cells when exposed to 3ug/ml of BrdUrd for 48 hours and 72 hours, each consist of two cell populations. The first cell population is distinct in that it's chromosomes are composed of half old and half new DNA, or from here on, referred to as hybrid chromosomes. The second distinct cell population has chromosomes composed of entirely new DNA, in that both chromatids contain BrdUrd substituted regions. The hybrid population of chromosomes in both the 48 and 72 hour exposed cells showed: 1.) differential fluorescence of sister chromatids (SCD) with extrene quenching of the doubly substituted and minor quenching of the singly substituted sister chromatid in both the 48 and 72 hour treated cells (Figure 3a, 3b, 8b); 2.) the differential staining of sister chromatids allows observation of sister chromatid exchanges (SCE), (Figure 3b), where brightly fluorescing regions have been exchanged. The 48 and 72 hour treated cells also contained a

second cell population which consisted of chromosomes where both sister chromatids were equally, double substituted. These chromosomes showed: 1.) highly fluorescing centromeric regions in both acrocentric and biarmed chromosomes (Figures 5a, 8c); 2.) greatly reduced fluorescence of both sister chromatids, with little or no interstitial or terminal areas of bright fluorescence

Subclone C_3471 which had been continuously cultured in lug/ml of BrdUrd exhibited: 1.) brightly fluorescing centrometric regions, in both acrocentric and biarmed chromosomes (Figures 6, 8d); 2.) generally dull fluorescence of both sister chromatids with no distinguishing interstitial or terminal areas.

Immunocytochemical Labeling of the Clone B_559 Control Cells

The metaphase chromosomes of B_559 cells which were cultured in the absence of BrdUrd, were not labeled by the PAP complex. These results were in agreement with theoretical aspects of immunocytochemical labeling. When there are no antigenetic sites present, there is no basis for an immunological reaction to occur.

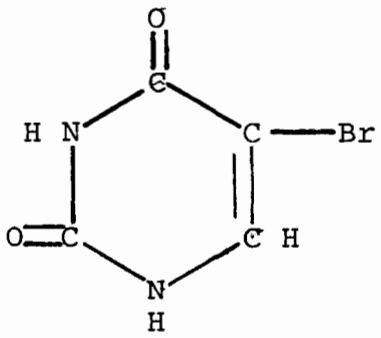
A further control was used as a check for antiserum specificity and cross reactivity. B_559 cells that had been exposed to BrdUrd, were reacted with the PAP reagents, omitting the goat-anti-rabbit bridge antibody. No labeling occured.

BrdUrd Incorporation Sites as Detected by the Unlabeled Antibody Enzyme Method

The hybrid population of chromosomes in clone B_559 cells exposed to BrdUrd for 48 and 72 hours showed: 1.) a heavy dark labeling on one sister chromatid, with slightly lighter label-

ing on the corresponding sister chromatid (Figure 4); 2.) the staining intensity of the centromeric regions varied for different chromosomes, with the majority being heavily labeled; 3.) some discontinuous staining of light and dark regions was present on the singly substituted sister chromatid. These results were consistent with the scheme of DNA replication, and substitution of BrdUrd.

The second population found present in the 48 and 72 hour treated cells showed: 1.) a discontinuous staining pattern of light and dark regions along the chromosomes. This pattern was variable between sister chromatids, with respect to the corresponding areas of heavy labeling; 2.) the staining intensity of the centromeric regions varied for different chromosomes, with the majority being heavily labeled (Figures 7a, 7b, 9c).



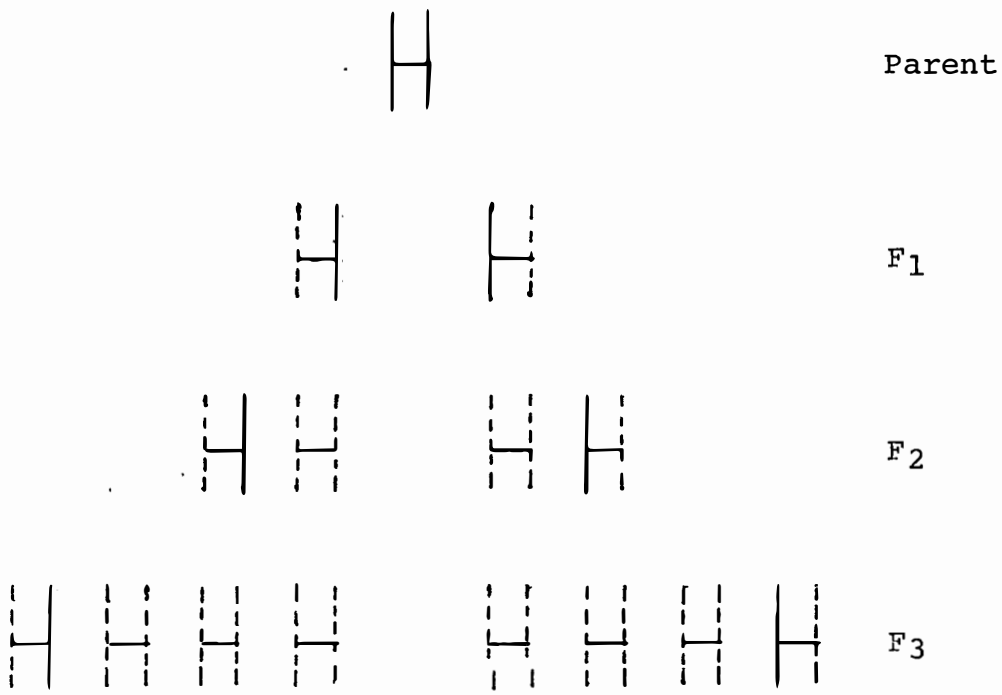
5-Bromouracil

5-Bromouracil is an analog of thymine in which the methyl group is replaced by a bromine atom at the site of the number 5 carbon.

Figure 1

The expected pattern of BrdUrd substitution per DNA strand according to the semiconservative replication of DNA. In the first generation (F_1), each daughter is half old and half new. The F_2 or second generation comprised two hybrid DNAs (half old, half new) and two new DNAs made up entirely of new strands. The F_3 generation comprises two hybrid DNAs and six entirely new DNAs.

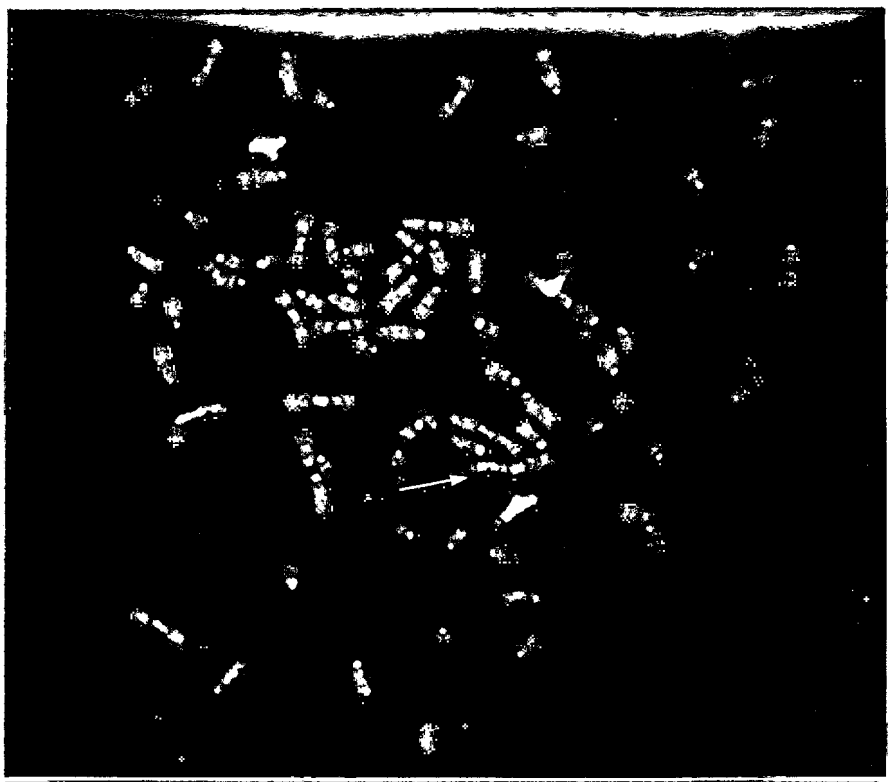
(——) DNA strand with no BrdUrd incorporation
(---) DNA strand where BrdUrd has been substituted for thymine.



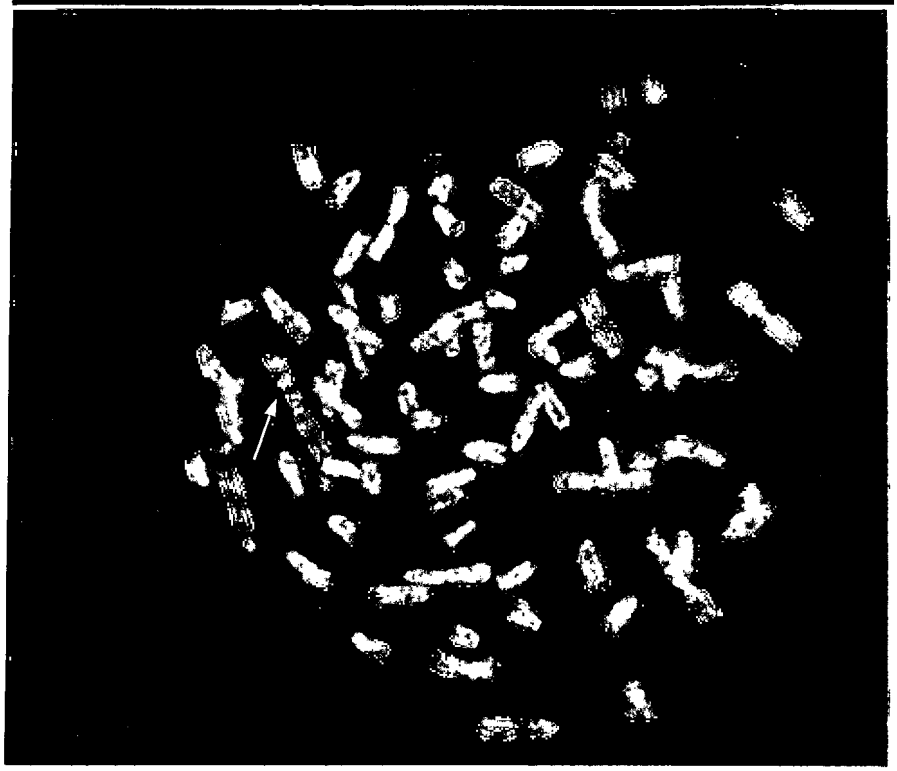
Figures 2a, 2b

33258 Hoechst stain of control cell chromosomes. $B_5$59 showing highly fluorescing centromeres of both acrocentric and bi-armed chromosomes and highly fluorescing regions of interstitial and terminal areas. Arrows indicate marker chromosomes. Magnifications = 2,700 X and 5,400 X, respectively.

2a



2b



3a

3b

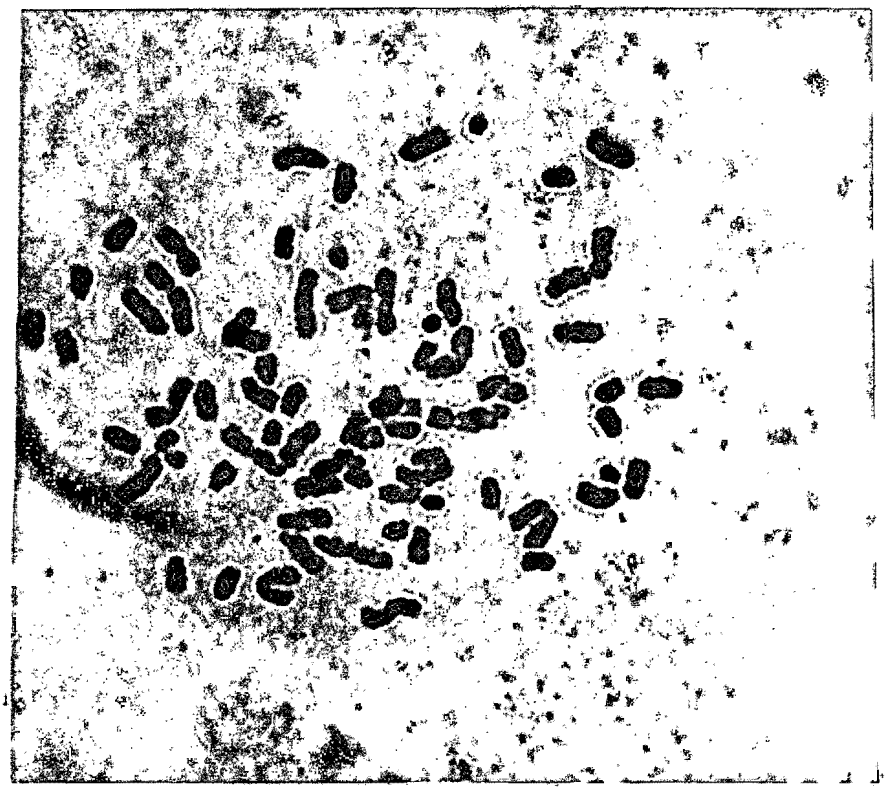


Figure 5a

33258 Hoechst stain of $B_5$59 cell chromosomes after 72 hours of exposure to 3ug/ml of BrdUrd, showing brightly fluorescing centromeric regions with generally reduced fluorescence of both sister chromatids.
Magnification = 4,050 X

Figure 5b

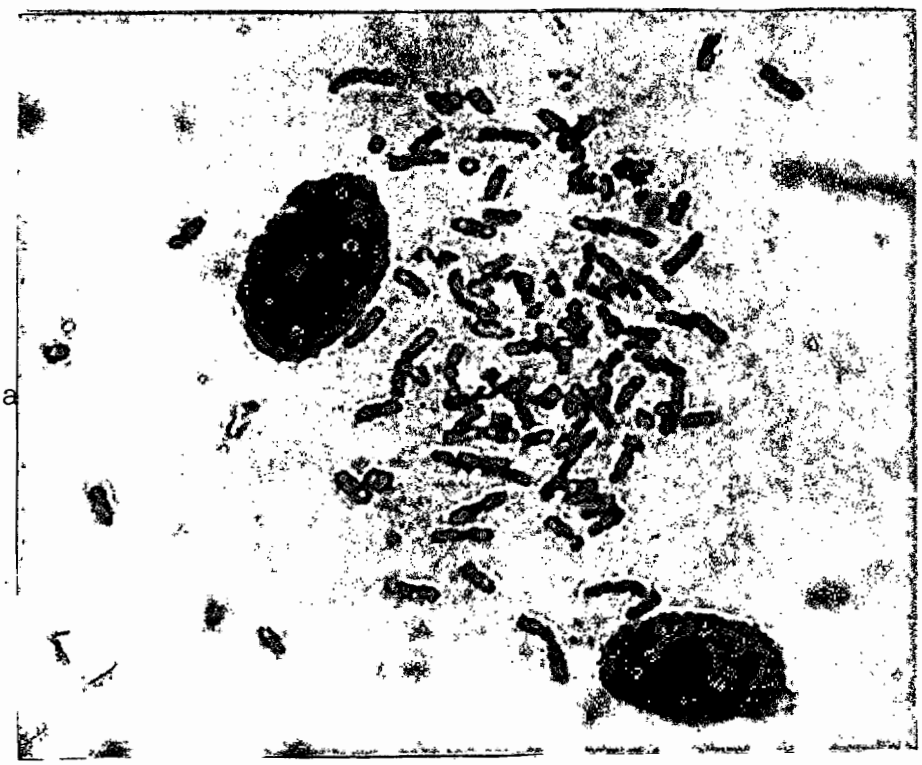
PAP labeling of $B_5$59 cell chromosomes after 72 hours of exposure to 3ug/ml of BrdUrd, showing a discontinuous pattern of light and dark regions along the chromosomes.
Magnification = 3,780 X

5a

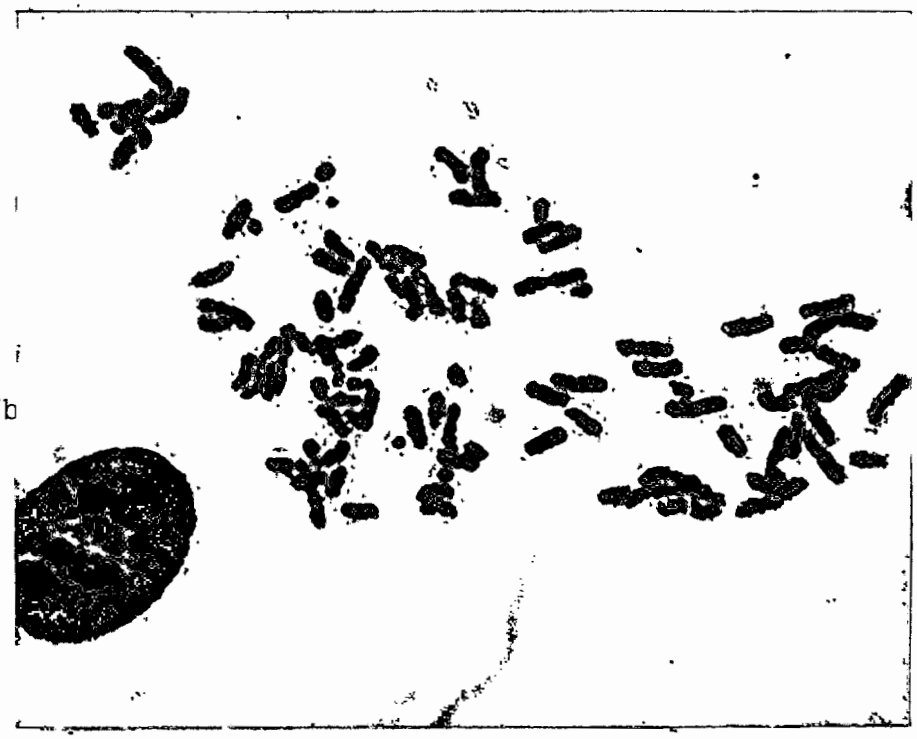
5b

Figures 7a, 7b

PAP labeling of $C_3$471 cell chromosomes, after continuous cultivation in lug/ml of BrdUrd, showing a discontinuous staining pattern of light and dark regions.
Magnifications = 3,510 X and 2,970 X, respectively.



7a



7b

Figure 8a

33258 Hoechst stain of control cells. Chromosomes $B_5$59.

Figure 8b

33258 Hoechst stain of $B_5$59 cell chromosomes after 72 hours exposure to 3ug/ml of BrdUrd. Note SCD and SCE.

Figure 8c

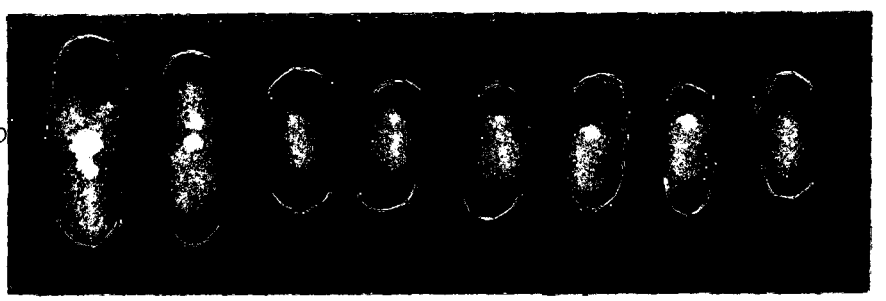
33258 Hoechst stain of $B_5$59 cell chromosomes after 72 hours exposure to 3ug/ml of BrdUrd. Note generally reduced fluorescence along the chromatids.

Figure 8d

33258 Hoechst stain of $C_3$471 cells after continuous exposure to lug/ml of BrdUrd. Note generally reduced fluorescnece along the chromatids.

8a

8b



8c

8d

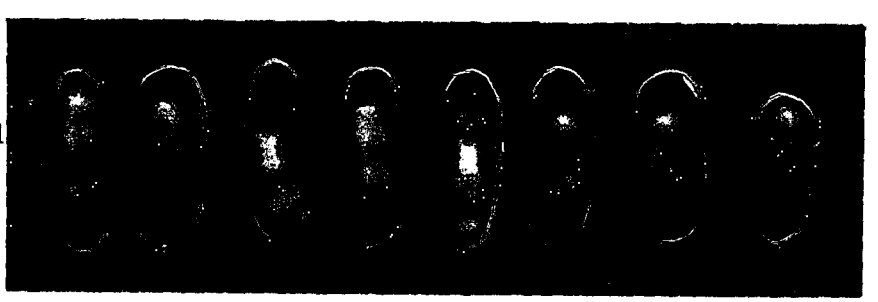


Figure 9a

PAP labeling of $B_5$59 cell chromosomes after 48 hours of exposure to 3ug/ml of BrdUrd. Note differential labeling of sister chromatids.

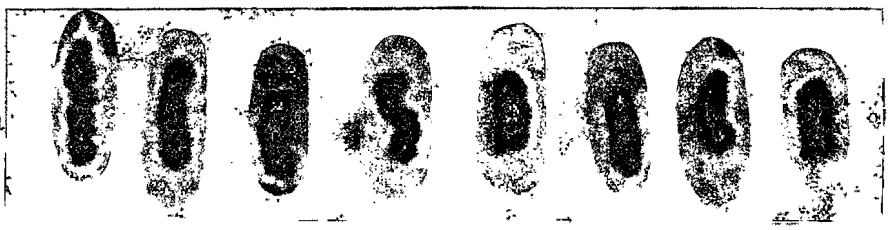
Figure 9b

PAP labeling of $B_5$59 cell chromosomes after 72 hours of exposure to 3ug/ml of BrdUrd. Note discontinuous labeling of sister chromatids.

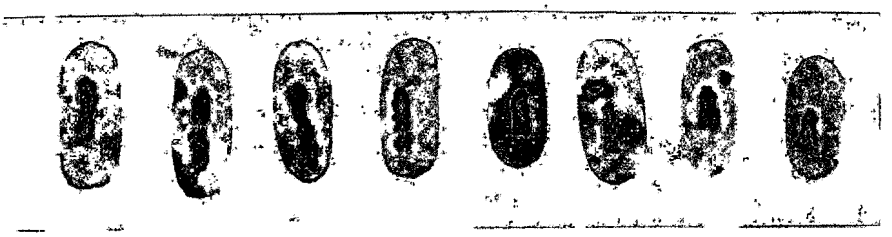
Figure 9c

PAP labeling of $C_3$471 cell chromosomes after continuous exposure to lug/ml of BrdUrd. Note discontinuous labeling of sister chromatids.

9a



9b



9c

of the sister chromatids. Therefore, it may be concluded that the BrdUrd once substituted into the normally bright fluorescence regions of the chromosomes, quenches the fluorescence intensity such that an overall dull fluorescence occurs.

The $C_3$471 cells, having been continuously cultured in BrdUrd at a concentration of lug/ml, also exhibits chromosome fluorescent patterns devoid of any unique regions along the chromatids, which is similar to those of the second population of both the 48 and 72 hour exposed $B_5$59 cells. However, without quantitative densitametric measurements it is difficult to determine any quantitative differences in BrdUrd incorporation patterns.

The Unlabeled Antibody Enzyme Method with the Peroxidase-Anti-peroxidase Complex

The data of the BrdUrd incorporation sites, as traced by the unlabeled antibody enzyme method has shown specific regions of heavy staining on both $C_3$471 cell chromosomes and those chromosomes of the $B_5$59 cells, that had BrdUrd incorporated into both sister chromatids. These immunologically labeled sites are clear and distinct, indicating specific labeling and preferential incorporation of BrdUrd into the regions so marked.

The immunocytochemical staining results from the dark brown color of the insoluble polymeric oxidation product of 3,3-diaminobenzedine tetrahydrochloride formed by the enzyme action. The precipitation occurs at the site of the linked enzyme-antibody-antigen complex, which is localized by this reaciton. The incorporated BrdUrd is the specific antigen, which in the single stranded denatured DNA, is free to react with the primary anti-

bodies. This technique is more sensitive than the Hoechst 33258 technique in that the reaction is more specific. Since the reaciton is enzyme mediated, more than one substrate product may be formed at any site of labeling, thus producing an amplification effect at the site of BrdUrd incorporation.

Aspects of BrdUrd Incorporation

The two techniques of staining have both been instrumental in locating regions of BrdUrd incorporation along the metaphase chromosomes studied. These regions appear at or near the centormeres as well as along the chromatids. Since the substitution of BrdUrd for thymidine is a completely random event, it would appear likely that substitution of BrdUrd for thymidine would preferentially occur at regions of high thymidine concentration over regions of low thymidine concentration. Therefore, by either limiting the exposure time to BrdUrd, or by limiting the concentration of BrdUrd, it is possible to control BrdUrd incorporation. These areas are indicative of areas rich in thymidine and adenine base pairs, in the untreated cells. As the amount of BrdUrd substitution increases, the probability that any one site or group of BrdUrd sensitive sites along the cell genome become substituted also increases. Silagi, 1976, has shown that increased amounts of BrdUrd substitutions affect an increase suppression of tumorigenicity and melanogenisis in the same $B_5$59 cell line when treated under the same conditions of BrdUrd exposure.

Significance of BrdUrd Incorporation and Heterochromatin

This data shows that the distinct interstitial and terminal regions of staining on the chromatids and centromeres of the

chromosomes studied may in fact represent areas of heterochromatin, repetitive sequences of DNA rich in thymine. Similar staining patterns of heterocrhomatin have been shown by Pasztor et al., 1976, in other tumorigenic long-term in vitro cultures derived from the original B16 mouse melanoma cells using centromeric (C) banding stains and in situ hybridization. Pardue and Gall, 1970, demonstrated that repetitive mouse satellite DNA is located in the heterochromatic region as localized by autoradiography.

Heterochromatin is referred to as that chromatin which remains compact during interphase of the cell cycle when the remander of the chromatin, the euchromatin, is more dispersed. The molecular mechanism which underlie this heterochromatinization is believed to control the degree of condensation and coiling of the chromosome and thus render the DNA inacessible for RNA transcription. It has been proposed by Brown, 1966, that there exists two types of heterochromatin; constitutive and facultative. Constitutive heterochromatin maintains its heterochromatin character at all times. The region adjacent to the centromeres of mouse chromosomes which maintain their heterochromatic character are regarded as constitutive. The constitutive heterochromatin is believed to be composed of highly repetitive base sequence which are not usually expressed. Mouse chromosomes have also been known to contain constitutive heterochromatic regions at their telomeres. In the case of facultative heterochromatin, it is believed that certain areas along the mouse chromosome can assume a heterochromatic or euchromatic state in response to a particular developmental stage. It

was alluded to that there may be other regions of the mouse chromosomes which contain constitutive heterchromatin and repetitive mouse satellite DNA. Britten and Davidson, 1969, suggested that in higher eukaryotes as much as 80% of DNA may be in repetitive sequences scattered among non-repetitive regions and having important regulatory functions. Strom and Dorfman, 1976, demonstrated preferential incorporation of BrdUrd into moderately repetitive regions, rather than into the unique sequences. They further suggest that the repetitive sequences upon substitution may be amplified and thus mediate major effects with small incorporation quantities,

Thus, unresolved is the precise molecular mechanism whereby BrdUrd-DNA mediates the preferential inhibition of differential functions, malignancy and viral induction. Since the effects are reversible in almost every case, mutation in the strict sense does not seem likely. The preferential effect of BrdUrd on synthesis of cell-specific products could reflect a selective effect on transcription. Schwartz and Kirsten, 1974, have found selective incorporation into DNA of rat embryo cells incubated in very low (10^{-7}M) concentration of BrdUrd, when fewer than 5% of the thymidine moieties are replaced. Schwartz et al., 1975, found a difference between distribution of ^{3}H-BrdUrd and ^{3}H-thymidine within repetitive and unique sequences of DNA. Turkington et al., 1971, hypothesized that selective inhibition of transcription could be due to substitution of BrdUrd in pyrimidine-rich initiator sites for transcription. Stellwagen and Tomkins, 1971, have proposed that the preferential effects could result from a generalized reduction in the rate of transcription

with differnetial labilities of certain mRNA's and their proteins. Whatever the exact mechanism of cellular control is, the Silagi mouse melanoma model system may exemplify such an instance of cellular regulation through heterochromatin, where a small amount of incorporation of BrdUrd into certain heterochromatic regions, mediates major effects of cell function.

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