EXTENSION OF BrdU-DYE ANALYSIS OF DNA REPLICATION AND SISTER CHROMATID EXCHANGE FORMATION TO *IN VIVO* SYSTEMS

(in vivo, BrdU-dye, DNA replication, sister chromatid exchange)

JAMES W. ALLEN, CHARLES F. SHULER, AND SAMUEL A. LATT

Clinical Genetics Division, Mental Retardation Program, Children's Hospital Medical Center and the Department of Pediatrics, Harvard Medical School
Boston, Massachusetts 02115

SUMMARY

BrdU-dye methodology was initially developed in tissue culture. Wide application of the technique for cytogenetic studies of DNA structure, replication and repair followed. Although the need for parallel in vivo studies was apparent, technical difficulties delayed the establishment of highly relevant BrdU-dye methods in intact mammals. Recently, BrdU-dye methods were adapted to in vivo rodent systems and the potential for diverse analyses of chromosome structure and function, comparable to those of in vitro analyses, demonstrated. In addition, the unique suitability of in vivo systems for studying replication kinetics and sister chromatid exchange formation in multiple tissues, inclusive of both somatic and germ cells, was shown. New in vivo protocols under development offer methodological simplicity and convenience of implementation. In vivo BrdU-dye techniques should thus afford an attractive alternative to in vitro systems for many cytogenetic studies.

INTRODUCTION

Most early cytogenetic studies in mammalian systems utilized spontaneously dividing cells isolated from intact animals (MAKINO 1951). Subsequent introduction of in vitro cell culture methods provided convenient, flexible and controlled systems for chromosome analyses. For example, tissue culture has become the predominant approach for implementing modern cytogenetic methods such as metaphase chromosome banding techniques which reveal detailed structural features of chromosomes (MILLER et al. 1973). Banding analysis relies upon tissue culture only for methodological simplicity; metaphase chromosomes isolated directly from animals can be similarly analyzed. In contrast, analysis of DNA replication, e.g. by autoradiography, requires administration of a radioactive nucleoside to replicating cells, a step most effectively carried out in the controlled environment of tissue culture.

Recently developed BrdU-dye techniques, which have extended and in some cases supplanted autoradiography for cytological analysis of DNA synthesis, have been extensively used for studies of chromosome organization and function in cultured cells. In these studies, BrdU incorporation is generally detected either by a reduction in the fluorescence efficiency of a chromosome stain such as 33258 Hoechst or by a reduction in Giemsa staining following photolytic degradation of substituted DNA. Control over the protocol of BrdU administration to cultured cells has been convenient for adapting the method to studies of DNA structure (LIN et al. 1974; LATT et al. 1974), replication (LATT 1973, 1974a, 1975; CROSSEN et al. 1975; STUBBLEFIELD 1975; MADAN et al. 1976), and repair (CHAGANTI et al. 1974; PERRY & WOLFF 1974; KORENBERG & FREEDLENDER 1974; KATO 1974; LATT 1974b, LATT et al. 1975a; SOLOMON & BOBROW 1975; BEEK & OBE 1975; RÜDIGER et al. 1976).

Results of in vitro studies utilizing BrdU-dye methods have indicated the need for parallel in vivo BrdU analyses (SAVAGE 1975). For example, intact animal studies would be most appropriate for determining the frequency and tissue distribution of sister chromatid exchange (SCE) induction following exposure to drugs which undergo host metabolic activation or detoxification. The establishment of in vivo BrdU-dye techniques which retain the relevance of mammalian systems, however, has been encumbered with technical difficulties in achieving sustained concentrations of the analogue during one or more DNA synthesis periods.

Recently, adaptation and utilization of BrdU-dye methodology for SCE analysis in somatic and germ cells of intact mice was reported (VOGEL & BAUKNECHT 1976; ALLEN & LATT 1976a). Suitability of in vivo BrdU-dye methodology for studies of tissue-specific replication kinetics and meiosis has also been demonstrated (ALLEN & LATT 1976b). Current development (ALLEN et al. In Press) of modifications in the mode of administering BrdU to animals holds promise for simplifying the in vivo procedure. The versatility and methodological straightforwardness of tissue culture is thereby retained with the in vivo approach, while a new level of relevance is gained.

BrdU-DYE METHODOLOGY

BrdU-dye methodology parallels autoradiography both in principle and potential application. The thymidine analogue BrdU, which is made available to replicating cells, can be subsequently detected in chromosomes, thereby providing information regarding DNA synthesis (LATT 1973). For example, BrdU can be clearly localized in metaphase chromosomes upon staining with the bisbenzimidazole dye 33258 Hoechst. BrdU suppresses dye fluorescence by reducing the quantum yield of bound dye molecules (LATT & WOHLLEB 1975). Other fluorescent dyes which have been used in similar fashion to detect BrdU incorporation include acridine orange (KATO 1974; DUTRILLAUX et al. 1974) and 4-6-diamidino-2-phenylindole (LIN & ALFI 1976). An immunofluorescent approach for detecting BrdU in chromosomes has also been reported (GRATZNER et al. 1974). Modified Giemsa protocols with (PERRY & WOLFF 1974) and without (KORENBERG & FREEDLENDER 1974) prior

33258 Hoechst treatment provide for permanent differential staining between sister chromatids asymmetrically substituted with BrdU. The Giemsa techniques apparently depend upon BrdU-dependent photolytic degradation of chromatin (GOTO et al. 1975).

Documentation of the semi-conservative distribution of newly replicated DNA between sister chromatids (TAYLOR et al. 1957) with BrdU-dye methodology has served to demonstrate that this approach possesses both convenience and high resolution which should facilitate broad application for cytogenetic analyses. Diverse additional studies can be undertaken with minor modifications in the protocol for exposing cells to BrdU. Cell culture experiments in which BrdU exposure is restricted to a fraction of one DNA synthesis period, one entire DNA synthesis period, or two DNA synthesis periods prior to cell harvest and staining illustrate the potential of the method for examining replication kinetics, structure, and SCE respectively.

IN VITRO STUDIES

The BrdU approach for studying chromosome replication kinetics is based upon differential stain intensity patterns which distinguish early and late-replicating chromosome regions. If BrdU is supplied to cells growing in culture for most of their DNA synthesis period but withheld (by media change) during terminal DNA synthesis, then regions last to replicate will incorporate thymidine rather than BrdU. 33258 Hoechst staining of metaphase chromosomes then highlights late-replication with bright fluorescence against a BrdU-suppressed fluorescence background. This is exemplified in Fig. 1 which shows mouse embryo cells with bright fluorescence characterizing late-replicating regions. Cell A, from a male, shows distinctive patterns of bright fluorescence characterizing the sex chromosomes, the centromeres and band-like autosomal regions. Cell B, from a female, shows late replication which is largely restricted to the sex chromosomes and centromeres. Late-replicating autosomal bands (Fig. 1A) highlighted with bright bisbenzimidazole fluorescence generally correspond to a subset of the structural bands (Q bands) which can be highlighted with the dye quinacrine. Generally, regions identified with bright quinacrine fluorescence correlate well with regions indicated by autoradiography to be late-replicating. However, optical techniques provide higher resolution and have permitted unequivocal demonstration of fluctuations in the timing of replication between homologues of a given cell and within individual chromosomes in different cells (LATT 1975). BrdU-dye methods also allow for detection of larger proportions of late-replicating X chromosomes in female cells and delineation of alternative patterns of replication in those chromosomes (WILLARD & LATT 1975; LATT et al. 1976).

If cells are allowed to complete a single DNA synthesis period in the presence of BrdU, 33258 Hoechst staining at the subsequent metaphase reveals structural features related to the unequal distribution of thymidine between complementary polynucleotide chains. In mouse cells (Fig.2) the chromosome arms

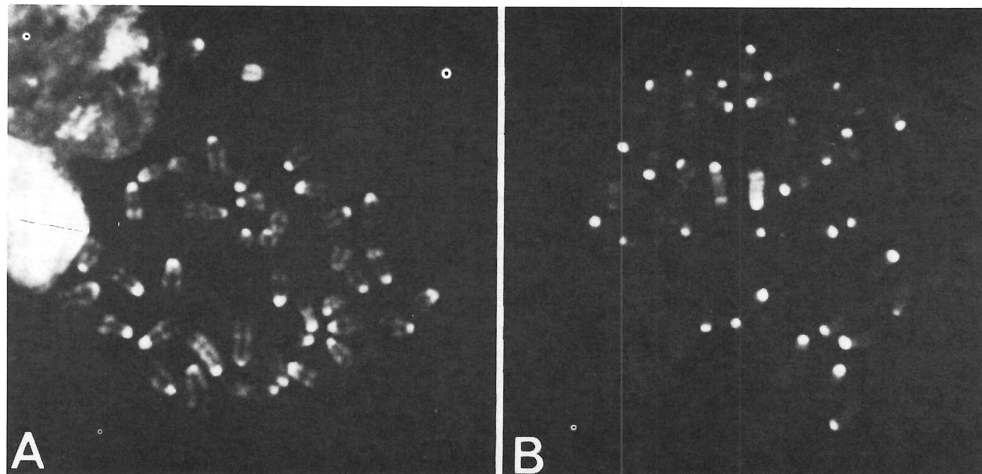

Figure 1. Fluorescence detection of late replication in mouse chromosomes (from MADAN, ALLEN, GERALD and LATT 1976). Mouse embryonic cells were grown in culture media with BrdU for 9 h followed by growth in media without BrdU for 9 h, and metaphase chromosomes were stained with 33258 Hoechst. In (A) a male cell shows bright fluorescence signifying late replication in the Y, the centromeres, and also band-like regions in the autosomes. In (B) a female cell shows bright fluorescence largely confined to the sex chromosomes and centromeres.

appear uniformly dull due to equivalent single-strand substitution; however, the centromeres exhibit lateral asymmetry signifying unequal thymidine content and BrdU substitution (LIN et al. 1974). Such fluorescence asymmetry is consistent with the maintenance of DNA polarity through the centromeres in metacentric mouse chromosomes. Studies in human lymphocyte cultures have similarly utilized first division fluorescence asymmetry to uncover regions of unequal thymidine distribution (LATT et al. 1974; ANGELL & JACOBS 1975; GALLOWAY & EVANS 1975) and, in a dicentric human Y chromosome, transcentromeric conservation of DNA polarity (LATT et al. 1974).

If cells undergo two complete cycles of BrdU incorporation,

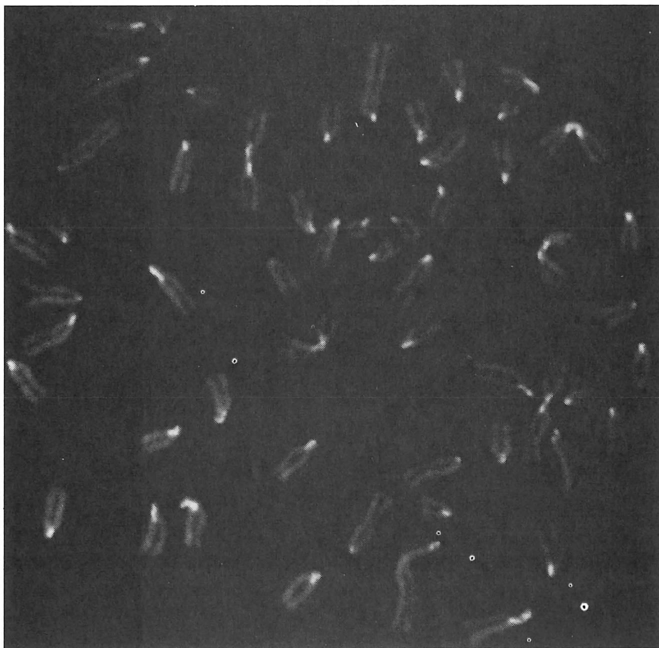

Figure 2. Mouse (RAG) fibroblast cells collected at the first metaphase division. Cells were harvested after 26 h growth in culture media containing BrdU. 33258 Hoechst staining reveals dull fluorescence in chromatid arms and lateral asymmetry of bright fluorescence in centromeres.

metaphase chromosomes stained with 33258 Hoechst will show sister chromatid differentiation due to unequal BrdU substitution (Fig.3). The dull chromatid reflects bifilar substitution, while the bright chromatid reflects unifilar substitution. The fluorescence contrast between chromatids permits detection of SCE's, which appear as reciprocal alterations in fluorescence along the chromatid lengths (Fig.4). Sister chromatid differentiation also characterizes some of the chromosomes from cells which undergo 3 or more divisions in BrdU. Accompanying chromosomes in these cells appear uniformly dull, reflecting bifilar substitution in both chromatids. The distinctiveness of first, second and later division BrdU-Hoechst patterns should be useful for studies of cell kinetics (LATT 1974a; LATT et al. 1975b; CRAIG-HOLMES & SHAW

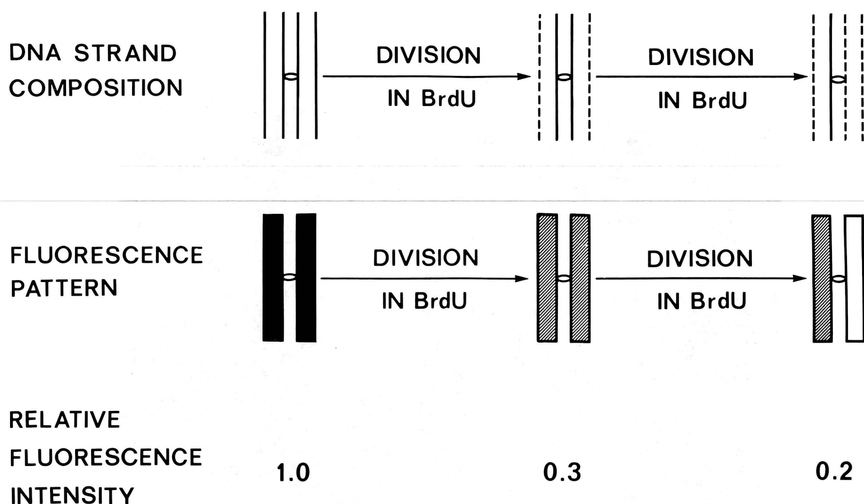

Figure 3. Diagrammatic representation of BrdU substitution for two successive DNA synthesis periods and resulting fluorescence patterns of first and second division metaphase cells after staining with 33258 Hoechst (from LATT 1974a). Unsubstituted DNA chains are represented by solid lines and BrdU-substituted chains by broken lines. Fluorescence of unsubstituted DNA is denoted as bright by black shading. First division chromosomes unifilarly substituted with BrdU exhibit a reduction in chromatid fluorescence by about two thirds (grey shading). Incorporation of BrdU for another cycle of replication results in one chromatid with unifilar substitution and one chromatid with bifilar substitution, the latter exhibiting further reduction in fluorescence as indicated by absence of shading. As a result, overall chromosome fluorescence is lower and sister chromatids are differentiated by fluorescence contrast.

1976; TICE et al. 1976; MILLER et al. 1976) in which proportions of cells replicating and/or timing of traverse through the cell cycle is of interest.

Perhaps the greatest impact of BrdU-dye methodology has

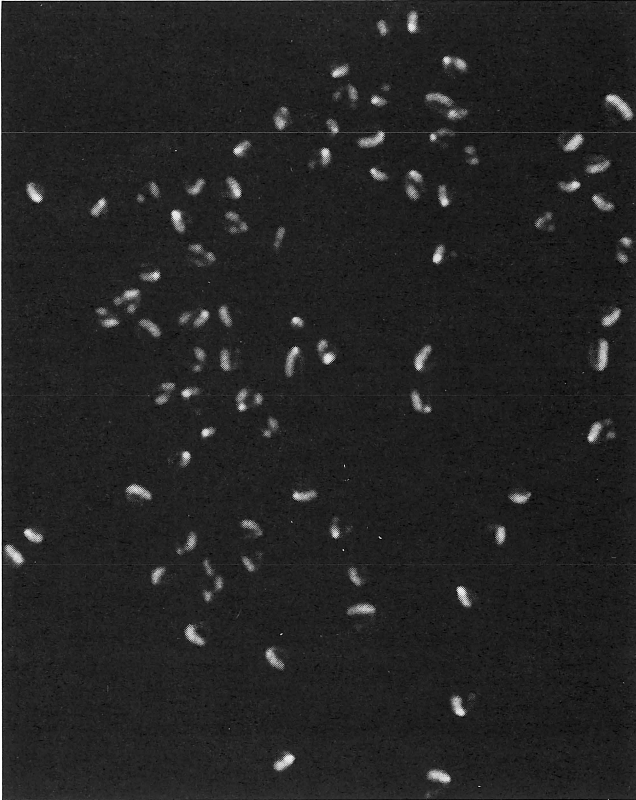

Figure 4. Mouse (3T3) fibroblast cells collected at the second metaphase division. Cells were grown in medium with BrdU and harvested after 45 h. Staining with 33258 Hoechst reveals sister chromatid differentiation and SCE.

been in chemical clastogen studies. SCE frequencies in second division cells have been found to constitute highly sensitive criteria for assessing the alteration of DNA by environmental agents (LATT et al. In Press). Although it is virtually impossible to use these techniques to exclude the hypothesis that baseline SCE frequencies themselves are induced by BrdU, significant increases in the SCE level have been shown to result from exposure to mitomycin C (Fig.5) (LATT 1974b), as well as a variety of other chemicals (PERRY & EVANS 1975), at doses associated with few or no gross chromosome aberrations. SCE has thus been interpreted as reflecting a form of DNA damage which is repaired and heretofore has gone undetected. Although the molecular basis of SCE is not entirely understood, certain correlations with chromosome breakage are evident. For example, mutagen-carcinogens such as 7,12-dimethylbenz (α) anthracene and 7, 8,12-trimethylbenz (α) anthracene have been shown to induce parallel distributions of breaks and SCE's along the chromosome lengths of cultured rat bone marrow cells (UEDA et al. 1976). Cultured Fanconi's anemia cells respond to mitomycin C with fewer than expected SCE's which, however, correlate with significantly increased chromatid breakage, about 50% of which occurs at sites of incomplete SCE (LATT et al. 1975a; LATT & JUERGENS 1977). It appears that, in some instances, chromosome aberrations may represent incomplete SCE's which are not repaired.

SCE analyses in cultured cells thus afford sensitive probing of chromosome damage and repair. Such analyses have proven useful in studies of disease states characterized by chromosome fragility (CHAGANTI et al. 1974; LATT et al. 1975a; WOLFF et al. 1977). They have also been used for examining mechanisms of DNA alteration (ROMMELAERE & MILLER-FAURES 1975; MOORE & HOLLIDAY 1976). Most often, the detection of chemical mutagens with BrdU-dye methods has employed in vitro SCE analysis. However, there are weaknesses inherent to in vitro systems for testing drug mutagenesis. A culture medium is an artificial approximation of the cell environment in intact animals, and compositional dissimilarities may affect cell responsiveness to drugs. In addition, the use of in vitro results to define a level of risk for man requires estimates of factors influencing drug concentration levels such as host activation or detoxification, drug binding to serum proteins, and tissue distribution of the drug.

Hybrid in vitro-in vivo BrdU-dye methods approach this problem, in limited fashion, by taking host metabolism into account. Somatic cells cultured in BrdU following extraction from drug-exposed humans (PERRY & EVANS 1975) or experimental animals (STETKA & WOLFF 1976a) provide an index of residual SCE induction; however, the extent of SCE formation occurring at peak exposure levels to the drug remains uncertain. Cells cultured in the presence of liver microsomal extract plus drug (AMES et al. 1975; STETKA & WOLFF 1976b) can reveal SCE induction by drugs which undergo liver microsomal modification by mixed function oxidases although they do not readily allow for analysis of drug metabolism by other pathways. Finally, neither hybrid system is very amenable to germ cell studies since these cells are classically difficult to maintain in culture.

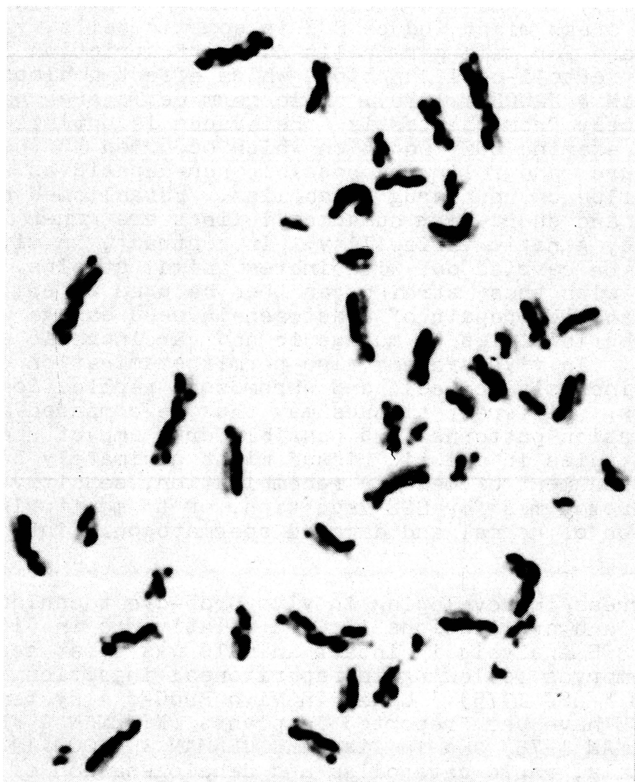

Figure 5. Human lymphocyte showing extensive SCE formation following exposure to mitomycin C. Cells were grown in medium with BrdU for 3 days. 0.1 μg/ml mitomycin C was added the third day. Staining with 33258 Hoechst, followed by photoillumination and Giemsa, reveals more than 50 SCE's. Control lymphocytes treated with BrdU, but not mitomycin C, have an average SCE frequency of about 12.

IN VIVO STUDIES

In vivo BrdU-dye systems in animals offer several advantages over in vitro systems for many cytogenetic studies. Tissue-specific comparisons might be made in mutagenesis trials to detect variations in drug accessibility or sensitivity. Different germ cell stages are known to be highly variable in their susceptibility to induced mutation (BATEMAN & EPSTEIN 1971). Also, some drugs might induce SCE in somatic cells, yet pose a lesser hazard for male germ cells due to restriction in passage across the sertoli-cell junctions which effect a blood-testis barrier (DYM & FAWCETT 1970). Male germ cells are readily prepared directly from the testis. Relevance is optimized by the ability to examine SCE induction which occurred at the time of drug exposure and following possible non-hepatic as well as hepatic influence upon drug metabolism. Established mammalian cell lines and short-term human cell lines are generally characterized by genetic variability. In contrast, in vivo SCE analyses can be carried out with inbred animal strains. Analysis of results with these strains can then be used to compare SCE formation and DNA repair of clastogen-induced damage with specific susceptibilities to mutagenic and carcinogenic effects of the agents. In vivo systems also permit examination of multiple functions inclusive of cell and chromosome replication kinetics and meiosis. Different tissues may thus be compared as to normal replication patterns and possible drug impact upon replication. Studies in meiotic tissue might ultimately be directed towards assessment of meiotic recombination, sensitivity of meiotic chromosomes for SCE induction, or to monitoring the differentiation of normal and damaged spermatogonia into spermatocytes.

Progress in developing in vivo BrdU-dye techniques has been slow and applications limited relative to in vitro methodology. SCE analysis in intact animals was first accomplished in chick embryos following intraperitoneal injection of BrdU in ovo (BLOOM & HSU 1975). Other in vivo BrdU-dye systems for analyzing SCE have been reported in plants (KIHLMAN & KRONBERG 1975; KIHLMAN 1975) and in fish (KLIGERMAN & BLOOM 1976). In adult mammals, rapid catabolism and dehalogenation of BrdU by the liver (BARRETT & WEST 1956) has made it technically difficult to provide animals with sustained exposure to BrdU so that levels of analogue remain sufficiently high for chromosome labeling over at least one DNA synthesis period. This problem has recently been overcome in rodents with protocols involving either multiple injections (VOGEL & BAUKNECHT 1976; ALLEN & LATT 1976a) or continuous infusion (SCHNEIDER et al. 1976; PERA & MATTIAS 1976). The use of hourly intraperitoneal injections over a time approximating one DNA synthesis period (in the tissue of interest), has been adapted for in vivo analyses of DNA structure, SCE formation, and replication kinetics in somatic and germ cells of mice (ALLEN & LATT 1976b). The remainder of the present paper is concerned with reviewing these studies and describing modified approaches under current development.

For studies of thymine asymmetry, cells are harvested at the first metaphase division following termination of the injec-

tions. As with the in vitro protocol, incorporation of BrdU for one complete DNA synthesis period reveals cells with uniformly dull fluorescence over unifilarly substituted chromatids and lateral asymmetry of fluorescence in centromeres (Fig.6). The asymmetrically bright centromeric regions reflect the thymidine-rich chain of mouse satellite DNA (PARDUE & GALL 1970) which serves as a template for less BrdU than that of its adenine-rich complement.

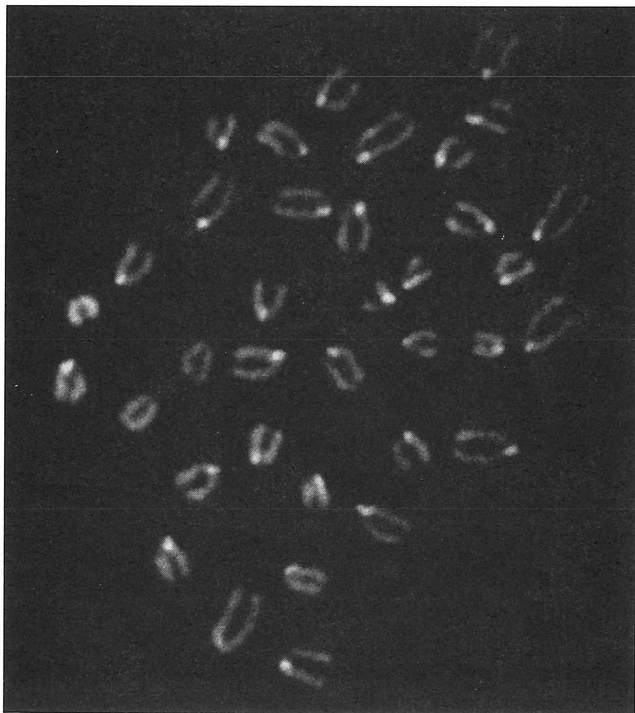

Figure 6. Mouse spermatogonial cell collected at the first metaphase division (from ALLEN and LATT 1976b). Mice were administered 14 hourly i.p. injections of BrdU and cells harvested 10 h later. After staining with 33258 Hoechst, centromeres fluoresce asymmetrically and chromatid arms exhibit dull fluorescence.

Cell harvest timed to coincide with the second mitotic metaphase following termination of injections provides chromosomes with sister chromatid differentiation. In contrast with the previous in vitro protocol in which cells undergo two successive divisions in BrdU, chromatid differentiation effected with the in vivo injection protocol is most conveniently achieved by allowing one replication in BrdU, followed by one replication in the absence of analogue (Fig.7). Contrast between chromatids consequently results from unifilar substitution in one chromatid and relative absence of BrdU in the other. The two forms of sister chromatid differentiation can be distinguished both by centromere patterns and by the appearance of third division cells. For example, dull fluorescence over half centromeres occurs in approximately three quarters of the chromatids after two rounds of BrdU incorporation, but in only one quarter of these sites if sequential cycles of BrdU and dT incorporation occur. Fig.8A denotes a mouse spermatogonial cell showing chromatid contrast and 3 SCE's. Low SCE frequencies (relative to in vitro systems) generally characterize cells studied with intact animal systems and enhance the attractiveness of in vivo protocols for SCE induction analyses. Third division cells following the in vivo injections protocol show ap-

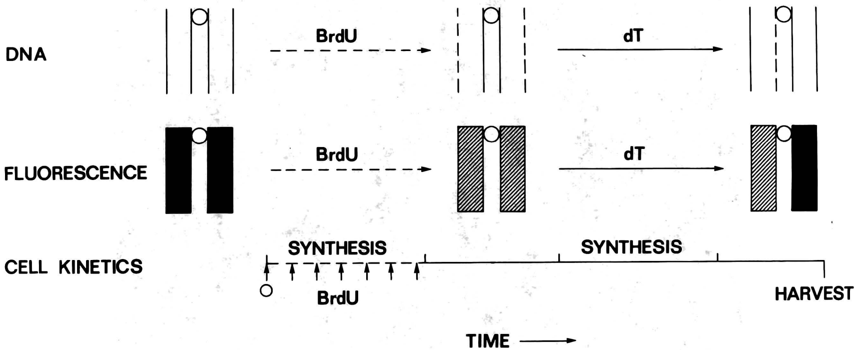

Figure 7. Diagrammatic representation of in vivo protocol for effecting sister chromatid differentiation. Unsubstituted DNA chains are represented by solid lines and BrdU-substituted chains by broken lines. 33258 Hoechst fluorescence is denoted as bright by black shading and dull by grey shading. Following BrdU incorporation for one DNA synthesis period, first division chromosomes are unifilarly substituted in each chromatid and exhibit dull fluorescence in chromatid arms. Subsequent replication without BrdU incorporation results in second division chromosomes with asymmetrical BrdU substitution. Bright fluorescence of unsubstituted chromatids contrasts with dull fluorescence of chromatids with single-strand substitution.

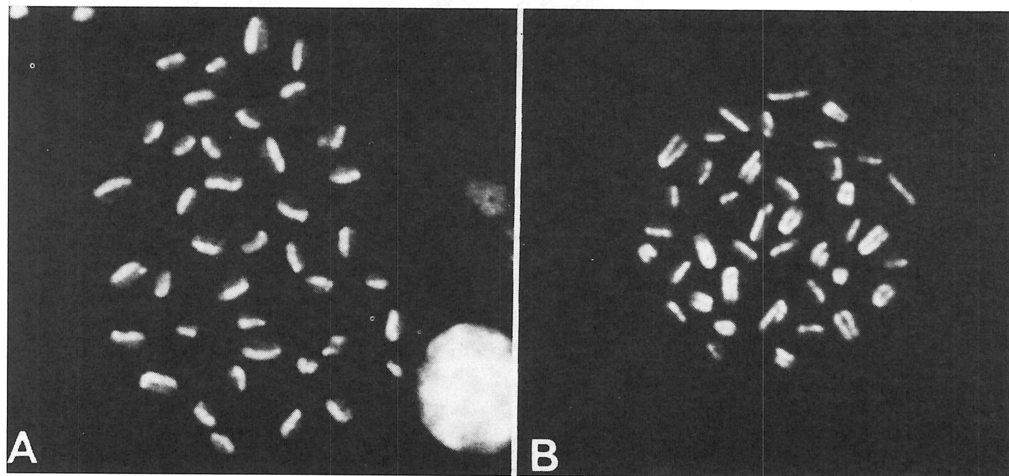

Figure 8. Mouse spermatogonial cells collected at the second and third metaphase divisions (from ALLEN and LATT 1976b). Mice were administered 14 hourly i.p. injections of BrdU and cells harvested 44 h (A) and 72 h (B) later. (A) After staining with 33258 Hoechst, sister chromatid differentiation and 3 SCE's are apparent. (B) 33258 Hoechst staining of third division cells reveals chromosomes which exhibit either sister chromatid differentiation or bright fluorescence in both chromatids.

proximately equal representation of two different kinds of chromosomes: those with chromatid contrast and those which are uniformly bright (Fig.8B). This is the expected pattern for cells which have undergone the first replication in BrdU and the remaining two replications in thymidine. The presumption that significant levels of BrdU are not available for a successive DNA synthesis period beyond termination of the injection series is thus supported.

We have applied the multiple injection protocol for in vivo SCE induction studies in second division spermatogonia and marrow cells (ALLEN & LATT 1976a,b). If a single intraperitoneal injection of 0.3 mg/kg mitomycin C is administered to mice at a time following the first cycle of DNA replication but prior to the second cycle, both spermatogonia (Fig.9) and marrow respond with marked SCE formation yet negligible increase in gross aberrations. Approximately threefold increases in the SCE levels occur in both tissues, despite differences in absolute SCE fre-

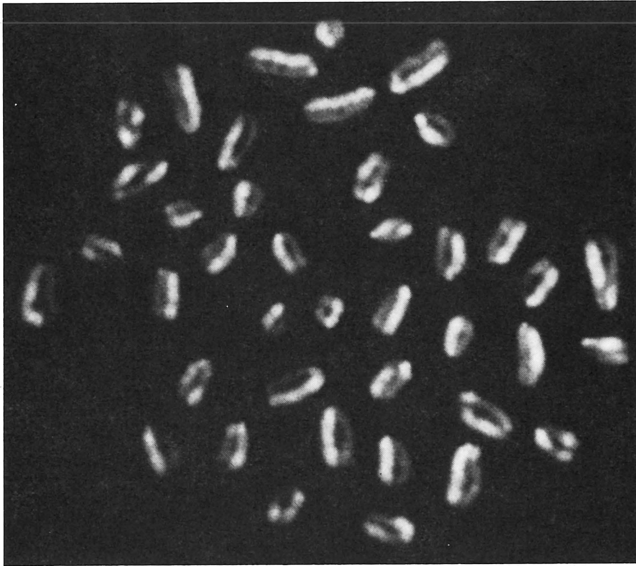

Figure 9. Extensive induction of SCE's in a mouse spermatogonial cell by mitomycin C (from ALLEN and LATT 1976a). This second division cell was treated as described in Figure 8A except that an i.p. injection of 0.5 mg/kg mitomycin C was given 12 h following termination of BrdU administration. Eighteen SCE's are evident.

quencies in controls (1.8/genome in spermatogonia and 3.4/genome in marrow). Analysis of SCE induction by drugs requiring host-activation was assessed in corresponding in vitro and in vivo trials with cyclophosphamide. Cyclophosphamide, a drug known to be converted in the liver to an active intermediate (CONNORS et al. 1974), was generally ineffective at inducing SCE's in mouse fibroblast and human lymphocyte cultures over a broad dose range. However, SCE levels in spermatogonia from mice injected with a 5 or 20 mg/kg cyclophosphamide dose between the first and second replications increased by three- and fivefold respectively.

A limitation of the in vivo protocols has been the labor involved in administering repeated injections or in maintaining a continuous infusion of BrdU over extended periods of time. This requirement is somewhat of a deterrent towards employing the system for large-scale mutagenesis testing. To circumvent this problem, we have explored the potential of various BrdU "depot" methods for simplifying BrdU administration. Our early studies in this area involved injection of mice with BrdU adsorbed to charcoal (RUSSEV & TSANEV 1975) to effect sustained release. Single injections were ineffective at producing second division chromatid contrast. Resort to multiple injections enabled second and third division marrow cells with acceptable chromatid contrast to be obtained. In the latter cells, fluorescence patterns resembled those of third division tissue culture cells in that approximately half of the chromosomes were uniformly dull (Fig.10A). Sustained release of BrdU over successive replication periods was thus accomplished. However, the requirement for multiple injections remained. In an alternative approach, subcutaneous implantation of various amounts of pure BrdU powder often provided clear contrast in second division cells; however, results were somewhat inconsistent and powder was concluded to be absorbed too rapidly.

We have recently reported excellent results obtained with a modified in vivo approach which relies upon the slow dissolution of a subcutaneously implanted BrdU tablet for sustained analogue exposure (ALLEN et al. In Press). BrdU "depot" administration in the form of tablets obviates the use of repeated injections or special restraining and infusion equipment and is typically accomplished in less than 5 minutes per animal. Third division marrow cells typically consist of approximately 50% chromosomes with uniform bright fluorescence (Fig.10B). This pattern confirms that BrdU incorporation is limited to the first replication. Fig.11 illustrates second division sister chromatid differentiation and SCE from control marrow and spleen cells harvested from the same animal. Comparable second division images may be obtained in thymus and spermatogonia tissues (Fig. 12). Effectiveness of the approach for in vivo analyses of SCE induction by cyclophosphamide in marrow and spleen has also been demonstrated. Fig. 13, A & B, illustrates SCE formation in second division marrow and spleen cells following i.p. injection of 20 mg/kg of drug timed to follow the first replication period. Average SCE induction in both tissues approximates a sevenfold increase over control levels (7.7 in marrow cells and 6.7 in spleen cells). Potential of the method for extension to other species and to other processes, i.e. meiosis, should be straightforward.

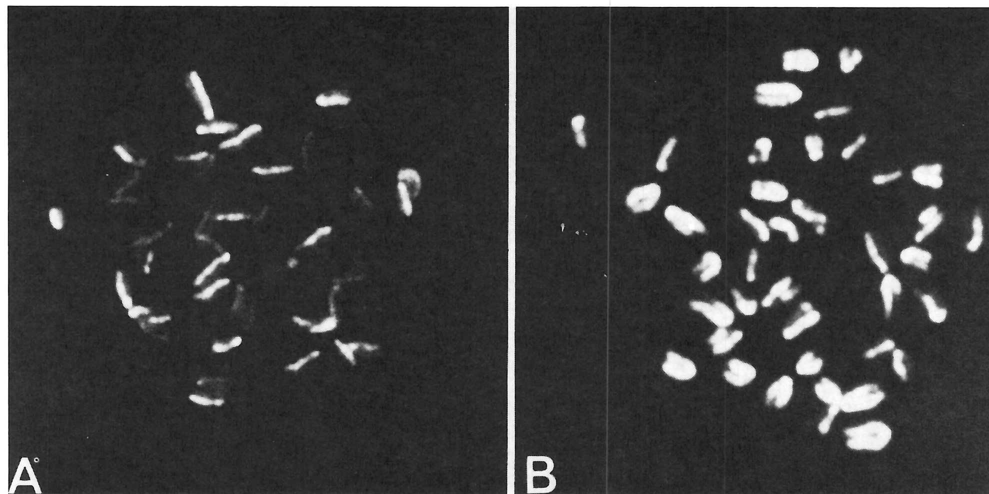

Figure 10. Third division, 33258 Hoechst-stained bone marrow cells harvested from mice given alternative modes of in vivo BrdU administration. Cell (A) is from a mouse which received 5 intraperitoneal injections of BrdU adsorbed to charcoal. Chromosomes reveal either sister chromatid differentiation or uniformly dull chromatid fluorescence, thereby indicating BrdU was incorporated for successive DNA synthesis periods. Cell (B) is from a mouse into which a BrdU tablet was implanted subcutaneously. Chromosomes exhibit either sister chromatid differentiation or uniformly bright chromatid fluorescence, a pattern indicative of BrdU incorporation over only the initial DNA synthesis period.

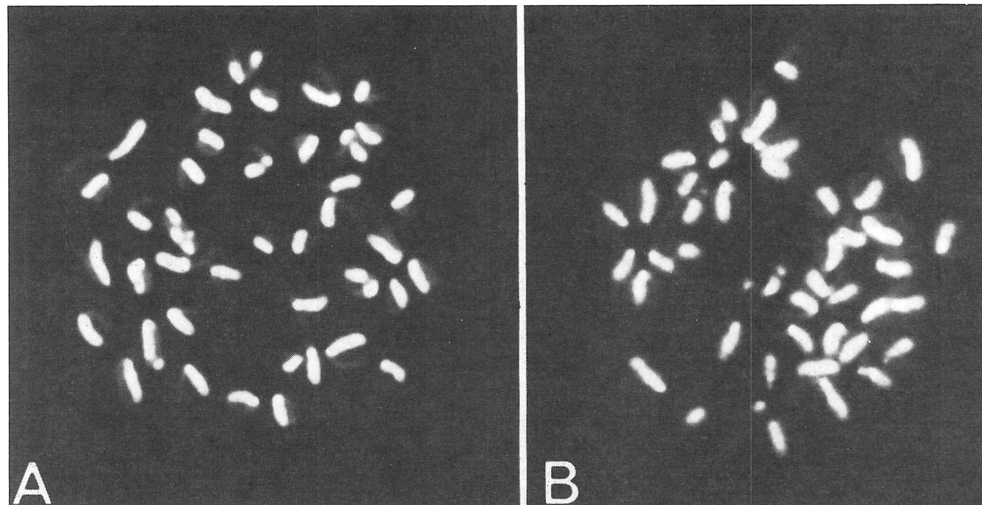

Figure 11. Bone marrow (A) and spleen (B) cells collected at the second metaphase division following subcutaneous implantation of a BrdU tablet (from ALLEN, SHULER, MENDES and LATT In Press). Cells were harvested from the same animal at 21 h and stained with 33258 Hoechst. Sister chromatid differentiation and 8 SCE's are apparent in both cells.

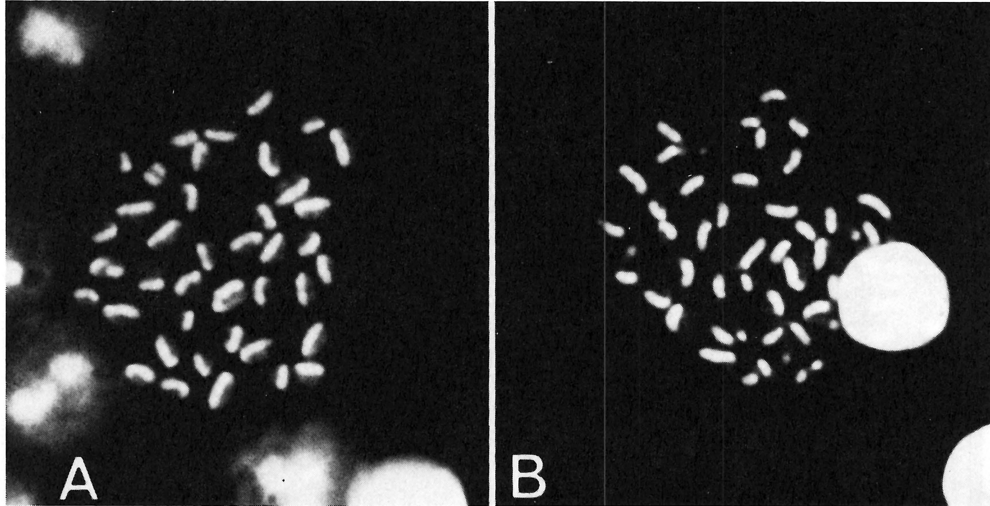

Figure 12. Spermatogonial (A) and thymus (B) cells at the second metaphase division following subcutaneous implantation of a BrdU tablet. (A) Spermatogonial cells were harvested at 58 h and stained with 33258 Hoechst. One SCE is apparent. (B) Thymus cells were harvested at 20 h. 33258 Hoechst staining reveals 8 SCE's.

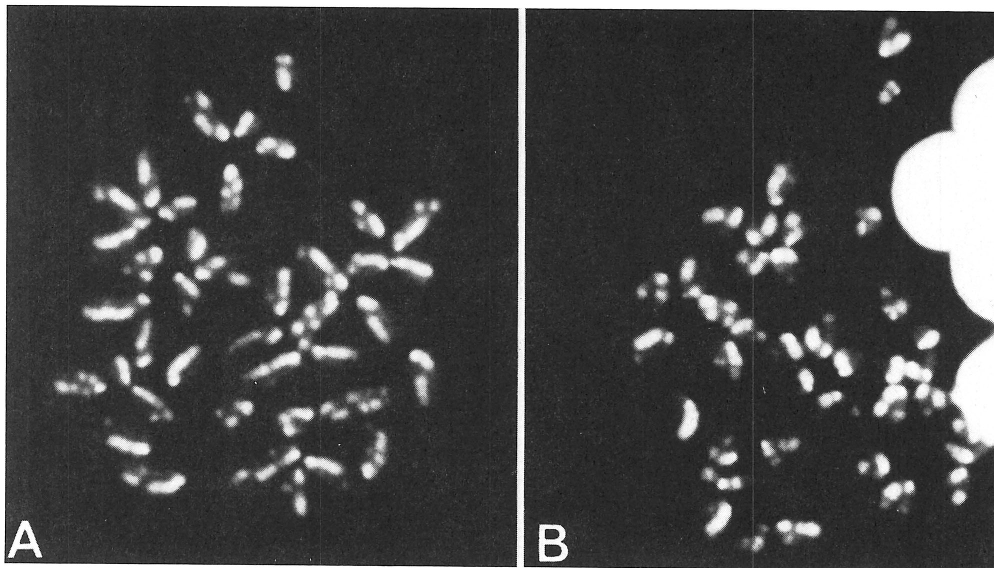

Figure 13. Extensive sister chromatid exchange induction by cyclophosphamide in bone marrow (A) and spleen (B) cells from a mouse implanted with a BrdU tablet (from ALLEN, SHULER, MENDES and LATT In Press). 20 mg/kg cyclophosphamide was injected at 8 h, and cells were harvested after an additional 17 h. 33258 Hoechst staining reveals approximately 60 SCE's in the bone marrow cell (A) and 40 SCE's in the spleen cell (B).

Although BrdU tablet methodology has, thus far, been confined in application to SCE analysis, the in vivo injections protocol has been examined for its effectiveness in studies of replication kinetics (ALLEN & LATT 1976b). Similar in principle to the in vitro replication kinetics protocol, if BrdU exposure and cell harvest are timed so that incorporation of base analogue occurs over part of one synthesis period which is completed with dT, bright 33258 Hoechst fluorescence can be used to highlight late-replicating chromosome regions. Fig.14A denotes a male mouse bone marrow cell showing late replication represented by bright sex chromosomes, centromeres, and banded regions in the chromosome arms. Fig.14B shows a mouse spermatogonial cell with late replication restricted, for the most part, to the Y chromosome. Preliminary observations of spermatogonia cells typically showing only the Y chromosome highlighted with 33258 Hoechst and marrow cells usually with autosomal bands ocurring coincident with the bright Y suggest that the Y may be relatively less late-labeling in marrow. A similar interpretation has been made from an earlier autoradiography study (TIEPOLO 1967). Should this apparent tissue specificity for timing of replication prove significant, these analyses might be extended with the in vivo system to include additional cell types.

If mice are injected with BrdU and harvest of spermatogonia is delayed for approximately two weeks, sister chromatid differentiation and SCE can be visualized in meiotic tissue (Fig.15B) (ALLEN & LATT 1976b). Thus far, unequivocal chromatid contrast has been restricted to sex chromosomes of primary spermatocytes. These cells may have differentiated from Type B spermatogonia which were terminally pulsed with BrdU during DNA synthesis. In most instances, chromatid differentiation characterized only the Y chromosome; however, cells with sister chromatid differentiation and SCE in the X chromosome were also observed. The occasional observation of centromere asymmetry in the X chromosome was noted to occur in either contralateral or parallel alignment with Y chromatid contrast, thus suggesting that maintenance of DNA polarity is not significant in the end-to-end association (HSU et al. 1971) of sex chromosomes in mice.

In conclusion, BrdU-dye techniques developed in intact animals have proven useful for diverse, high resolution analyses involving DNA synthesis. These methods have been extended from in vitro systems without loss of sensitivity and applicability, and versatility and relevance have been gained. Recent modifications in the mode of administering BrdU to animals lend methodological simplicity to the procedure. Thus the in vivo approach should provide an attractive alternative to cell culture systems for a variety of cytogenetic studies.

Practical applications might include utilization of in vivo systems for large-scale host-mediated assays of SCE induction by environmental mutagens. Enhancement of such studies by combining them with methods for detecting SCE automatically (ZACK et al. 1976) or biochemically appears feasible. Also, extension of the in vivo analyses to additional tissues, i.e., liver and fetal tissues, and to other species appears straightforward. The in vivo approach may also help clarify the possible

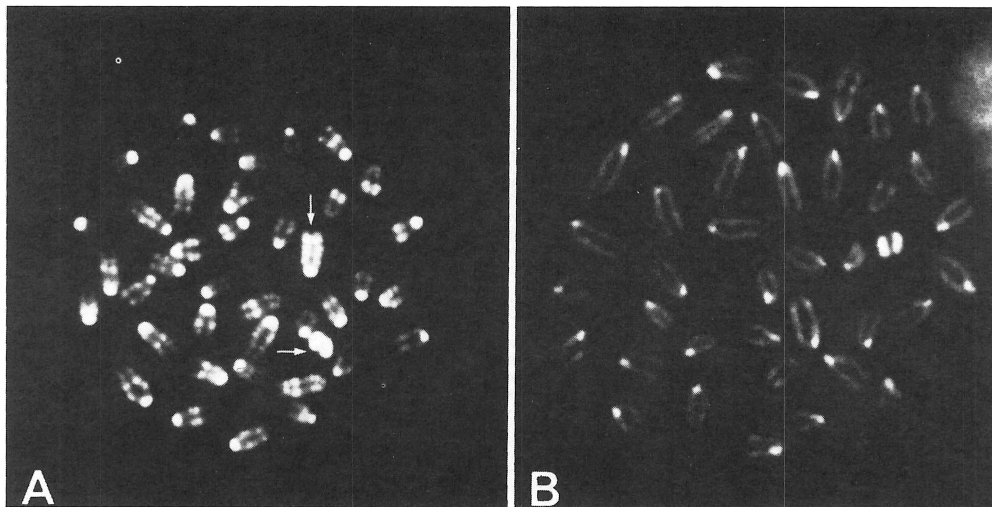

Figure 14. Late replication in male mouse cells (from ALLEN and LATT 1976b). BrdU administration and cell harvest were timed to provide cells with BrdU for part of one DNA synthesis period, which was completed in the absence of BrdU. Cell (A) represents a bone marrow cell in which late-replicating regions, highlighted by bright 33258 Hoechst fluorescence, include sex chromosomes (arrows), centromeres, and autosomal band-like areas. Cell (B) reflects a spermatogonial cell with bright 33258 Hoechst fluorescence primarily in the Y chromosome.

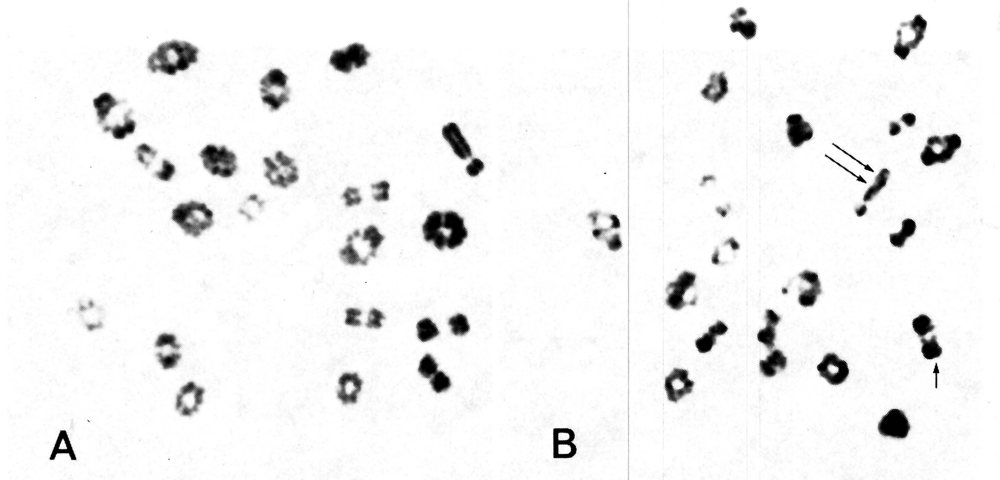

Figure 15. Mouse, first meiotic metaphase cells. Cell (A) was collected from a control mouse which was not administered any BrdU. Cell (B) (from ALLEN and LATT 1976b) was collected from a mouse which, 14 days earlier, received 14 hourly i.p. injections of BrdU. Following 33258 Hoechst staining, photoillumination and Giemsa staining, sister chromatid differentiation is not apparent in cell (A); however, in cell (B) it is notable in the sex chromosomes, which are aligned end-to-end. Two SCE's are apparent in the X chromosome (long arrows). There is also a suggestion of contrast between sister chromatids in at least one autosomal bivalent (short arrow).

implications of SCE for carcinogenesis and teratogenesis. To this end, replication kinetics and SCE formation might be examined in tumor cells and stem cells isolated from intact animals. Continuing meiotic studies are directed to the eventual elucidation of "genetic" recombination. Visualization of meiotic chromatid interchange would be fundamentally significant for innumerable basic and applied genetic studies. In vivo BrdU-dye methodology thus has a broad potential, both for extending many in vitro cytogenetic studies and for providing new and otherwise inaccessible information.

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LITERATURE CITED

ALLEN, J. W. and S. A. LATT 1976a Analysis of sister chromatid exchange formation in vivo in mouse spermatogonia as a new test system for environmental mutagens. Nature 260: 449-451.

ALLEN, J. W. and S. A. LATT 1976b In vivo BrdU-33258 Hoechst analysis of DNA replication kinetics and sister chromatid exchange formation in mouse somatic and meiotic cells. Chromosoma 58: 325-340.

ALLEN, J. W., C. F. SHULER, R. W. MENDES and S. A. LATT (In Press) A simplified technique for in vivo analysis of sister chromatid exchanges using 5-bromodeoxyuridine tablets. Cytogenet. and Cell Genet.

AMES, B. N., J. McCANN and E. YAMASAKI 1975 Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. Mutat. Res. 31: 347-364.

ANGELL, R. R. and P. A. JACOBS 1975 Lateral asymmetry in human constitutive heterochromatin. Chromosoma 51: 301-310.

BARRETT, H. W. and R. A. WEST 1956 Dehalogenation of substituted pyrimidines in vivo. J. Am. Chem. Soc. 78: 1612-1615.

BATEMAN, A. J. and S. S. EPSTEIN 1971 Dominant lethal mutations in mammals. pp. 541-568. Chemical Mutagens, 2. (Hollaender, A., Ed.) Plenum Press, New York.

BEEK, B. and OBE, G. 1975 The human leukocyte test system VI. The use of sister chromatid exchanges as possible indicators for mutagenic activities. Humangenetik 29: 127-134.

BLOOM, S. E. and T. C. HSU 1975 Differential fluorescence of sister chromatids in chicken embryos exposed to 5-bromodeoxyuridine. Chromosoma 51: 261-267.

CHAGANTI, R. S. K., S. SCHONBERG and J. GERMAN 1974 A manyfold increase in sister chromatid exchanges in Bloom's

syndrome lymphocytes. Proc. Nat. Acad. Sci. U.S. 71: 4508-12.

CONNORS, T. A., P. J. COX, P. B. FARMER, A. B. FOSTER and M. JARMAN 1974 Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochem. Pharmacol. 23: 115-129.

CRAIG-HOLMES, A. P. and M. W. SHAW 1976 Cell cycle analysis in asynchronous cultures using the BudR-Hoechst technique. Exp. Cell Res. 99: 79-87.

CROSSEN, P. E., S. PATHAK and F. E. ARRIGHI 1975 A high resolution study of the DNA replication patterns of Chinese hamster chromosomes using sister chromatid differential staining technique. Chromosoma 52: 339-347.

DUTRILLAUX, M. B., A. M. FOSSE, M. PRIEUR and J. LEJEUNE 1974 Analyse des échanges de chromatides dans les cellules somatiques humaines, traitement au BUDR et fluorescence bicolore par l'acridine orange. Chromosoma 48: 327-340.

DYM, M. and D. W. FAWCETT 1970 The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biol. Reprod. 3: 308-326.

GALLOWAY, S. M. and H. J. EVANS 1975 Asymmetrical C-bands and satellite DNA in man. Exper. Cell Res. 54: 454-459.

GOTO, K., T. AKEMATSU, H. SHIMAZU and T. SUGIYAMA 1975 Simple differential Giemsa staining of sister chromatids after treatment with photosensitive dyes and exposure to light and the mechanism of staining. Chromosoma 53: 223-230.

GRATZNER, H. G., A. POLLACK, D. J. INGRAM and R. C. LIEF 1976 Detection of deoxyribonucleic acid replication in single cells and chromosomes by immunologic techniques. J. Histochem. Cytochem. 24: 34-39.

HSU, T. C., J. E. K. COOPER, M. L. MACE, Jr., and B. R. BRINKLEY 1971 Arrangement of centromeres in mouse cells. Chromosoma 34: 73-87.

KATO, H. 1974 Spontaneous sister chromatid exchanges detected by a BudR-labelling method. Nature 251: 70-72.

KIHLMAN, B. A. 1975 Sister chromatid exchanges in Vicia faba II. Effects of thiotepa, caffeine, and 8-ethoxycaffeine on the frequency of SCE's. Chromosoma 51: 11-18.

KIHLMAN, B. A. and D. KRONBERG 1975 Sister chromatid exchanges in Vicia faba. I. Demonstration by a modified fluorescent plus Giemsa (FPG) technique. Chromosoma 51: 1-10.

KLIGERMAN, A. D. and S. E. BLOOM 1976 Sister chromatid differentiation and exchanges in adult mudminnows (Umbra limi) after in vivo exposure to 5-bromodeoxyuridine. Chromosoma 56: 101-109.

KORENBERG, J. and E. FREEDLENDER 1974 Giemsa technique for detection of sister chromatid exchanges. Chromosoma 48: 355-60.

LATT, S. A. 1973 Microfluorometric detection of DNA replication in human metaphase chromosomes. Proc. Nat. Acad. Sci. U.S. 70: 3395-3399.

LATT, S. A. 1974a Microfluorometric analysis of deoxyribonucleic acid replication kinetics and sister chromatid exchanges in human chromosomes. J. Histochem. Cytochem. 22: 478-491.

LATT, S. A. 1974b Sister chromatid exchanges, indices of human chromosome damage and repair: detection by fluorescence

and induction by mitomycin C. Proc. Nat. Acad. Sci. U.S. 71: 3162-66.

LATT, S. A. 1975 Fluorescence analysis of late DNA replication in human metaphase chromosomes. Somat. Cell Genet. 1: 293-321.

LATT, S. A. and J. C. WOHLLEB 1975 Optical studies of the interaction of 33258 Hoechst with DNA, chromatin and metaphase chromosomes. Chromosoma 52: 297-316.

LATT, S. A. and L. A. JUERGENS 1977 Determinants of sister chromatid exchange frequencies in human chromosomes. pp. 217-236. Population Cytogenetics. (Hook, E. B. and I. H. Porter, Eds.) Academic, New York.

LATT, S. A., R. L. DAVIDSON, M. S. LIN and P. S. GERALD 1974 Lateral asymmetry in the fluorescence of human Y chromosomes stained with 33258 Hoechst. Exper. Cell Res. 87: 425-429.

LATT, S. A., G. STETTEN, L. A. JUERGENS, G. R. BUCHANAN and P. S. GERALD 1975a The induction by alkylating agents of sister chromatid exchanges and chromatid breaks in Fanconi's anemia. Proc. Nat. Acad. Sci. U.S. 72: 4066-4070.

LATT, S. A., G. STETTEN, L. A. JUERGENS, H. F. WILLARD and C. D. SCHER 1975b Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence. J. Histochem. Cytochem. 23: 493-505.

LATT, S. A., H. F. WILLARD and P. S. GERALD 1976 BrdU-33258 Hoechst analysis of DNA replication in human lymphocytes with supernumerary or structurally abnormal X chromosomes. Chromosoma 57: 135-153.

LATT, S. A., J. W. ALLEN, W. E. ROGERS and L. A. JUERGENS (In Press) In vitro and in vivo analysis of sister chromatid exchange formation. Mutat. Res.

LIN, M. S. and O. S. ALFI 1976 Detection of sister chromatid exchanges by 4'-6-diamidino-2-phenylindole fluorescence. Chromosoma 57: 219-225.

LIN, M. S., S. A. LATT and R. L. DAVIDSON 1974 Microfluorometric detection of asymmetry in the centromeric region of mouse chromosomes. Exper. Cell Res. 86: 392-395.

MADAN, K., J. W. ALLEN, P. S. GERALD and S. A. LATT 1976 Fluorescence analysis of late DNA replication in mouse metaphase chromosomes using BrdU and 33258 Hoechst. Exper. Cell Res. 99: 438-444.

MAKINO, S. 1951 Studies on the murine chromosomes. V. A study of the chromosomes in Apodemus, especially with reference to the sex chromosomes in meiosis. J. Morphol. 88: 93-126.

MILLER, O. J., D. A. MILLER and D. WARBURTON 1973 Application of new staining techniques to the study of human chromosomes. Prog. Med. Genet. 9: 1-47.

MILLER, R. C., M. M. ARONSON and W. W. NICHOLS 1976 Effects of treatment on differential staining of BrdU labeled metaphase chromosomes: three-way differentiation of M3 chromosomes. Chromosoma 55: 1-11.

MOORE, P. D. and R. HOLLIDAY 1976 Evidence for the formation of hybrid DNA during mitotic recombination in Chinese hamster cells. Cell 8: 573-579.

PARDUE, M. L. and J. G. GALL 1970 Chromosomal localization of mouse satellite DNA. Science 168: 1356-1358.

PERA, F. and P. MATTIAS 1976 Labelling of DNA and differential sister chromatid staining after BrdU treatment in vivo. Chromosoma 57: 13-18.

PERRY, P. and S. WOLFF 1974 New Giemsa method for the differential staining of sister chromatids. Nature 251: 156-158.

PERRY, P. and H. J. EVANS 1975 Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature 258: 121-124.

ROMMELAERE, J. and A. MILLER-FAURÈS 1975 Detection by density equilibrium centrifugation of recombinant-like DNA molecules in somatic mammalian cells. J. Molec. Biol. 98: 195-218.

RUDIGER, H. W., F. KOHL, W. MANGELS, P. VON WICHERT, C. R. BARTRAM, W. WOHLER and E. PASSARGE 1976 Benzpyrene induces sister chromatid exchanges in cultured human lymphocytes. Nature 262: 290-292.

RUSSEV, G. and R. TSANEV 1975 Application of precursors adsorbed on activated charcoal for labeling of mammalian DNA in vivo. pp. 115-122. Methods in Cell Biology, IX. (Prescott, D. M., Ed.) Academic Press, New York.

SAVAGE, R. K. 1975 Chromosomal aberrations as tests for mutagenicity. Nature 258: 103-104.

SCHNEIDER, E. L., J. R. CHAILLET and R. R. TICE 1976 In vivo BudR labeling of mammalian chromosomes. Exp. Cell Res. 100: 396-398.

SOLOMON, E. and M. BOBROW 1975 Sister chromatid exchanges--a sensitive assay of agents damaging human chromosomes. Mutat. Res. 30: 273-278.

STETKA, D. G. and S. WOLFF 1976a Sister chromatid exchange as an assay for genetic damage induced by mutagen-carcinogens. I. In vivo test for compounds requiring metabolic activation. Mutat. Res. 41: 333-342.

STETKA, D. G. and S. WOLFF 1976b Sister chromatid exchange as an assay for genetic damage induced by mutagen-carcinogens. II. In vitro test for compounds requiring metabolic activation. Mutat. Res. 41: 343-350.

STUBBLEFIELD, E. 1975 Analysis of the replication pattern of Chinese hamster chromosomes using 5-bromodeoxyuridine suppression of 33258 Hoechst fluorescence. Chromosoma 53: 209-221.

TAYLOR, J. H., P. S. WOODS and W. L. HUGHES 1957 The organization and duplication of chromosomes as revealed by autoradiographic studies using tritium-labelled thymidine. Proc. Nat. Acad. Sci. U.S. 43: 122-128.

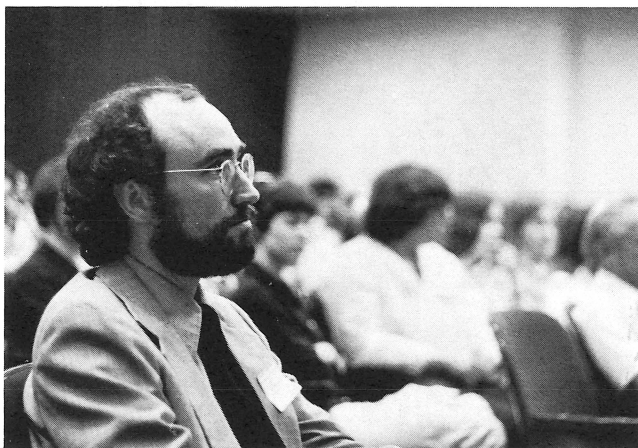
TICE, R., E. L. SCHNEIDER and J. M. RARY 1976 The utilization of bromodeoxyuridine incorporation into DNA for the analysis of cellular kinetics. Exp. Cell Res. 102: 232-236.

TIEPOLO, L., M. FRACCARO, M. HULTEN, J. LINDSTEN, A. MANNINI and P.-M. L. MING 1967 Timing of sex chromosome replication in somatic and germ-line cells of the mouse and the rat. Cytogenetics 6: 51-66.

UEDA, N., H. UENAKA, T. AKEMATSU and T. SUGIYAMA 1976 Parallel distribution of sister chromatid exchanges and chromosome aberrations. Nature 262: 581-583.

VOGEL, W. and T. BAUKNECHT 1976 Differential chromatid stain-

ing by in vivo treatment as a mutagenicity test system. Nature 260: 448-449.
WILLARD, H. F. and S. A. LATT 1976 Analysis of deoxyribonucleic acid replication in human X chromosomes by fluorescence microscopy. Amer. J. Hum. Genet. 28: 213-227.
WOLFF, S., B. RODIN and J. E. CLEAVER 1977 Sister chromatid exchanges induced by mutagenic carcinogens in normal and xeroderma pigmentosum cells. Nature 265: 347-349.
ZACK, G. W., J. A. SPRIET, S. A. LATT, G. H. GRANLUND and I. T. YOUNG 1976 Automatic detection and localization of sister chromatid exchanges. J. Histochem. Cytochem. 24: 168-177.



Dr. Allen at the Symposium

Part of the audience in the Student Union Auditorium