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Werner G. Bergen
Major professor

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DEVELOPMENT OF A HIGH-RESOLUTION BANDING TECHNIQUE FOR BOVINE CHROMOSOMES

By

Jonathan Mark Phillips

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

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ABSTRACT

DEVELOPMENT OF A HIGH-RESOLUTION BANDING TECHNIQUE FOR BOVINE CHROMOSOMES

By

Jonathan Mark Phillips

Precise characterization of chromosomes is being used extensively to resolve anomalies associated with genetic disorders. Although techniques to achieve high-resolution banding in human chromosomes are highly developed, these techniques have not been adapted for use in domestic animal species. The objective of this study was to develop a high-resolution chromosome banding technique. A technique proven to be valid for preparing human chromosomes was modified for use in the bovine species, _Bos taurus_. This technique employs amethopterin, an inhibitor of deoxyribonucleic acid (DNA) synthesis followed by rescue of cells with thymidine and collection of chromosomes at a critical time early in mitosis. Optimum duration of treatments was determined to be 16.5 and 4.75 h for amethopterin and thymidine, respectively. The developement of this technique will allow for more detailed studies of bovine chromosomes.

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I would like to thank Dr. W.G Bergen for serving as my major professor, and for handling me in a manner resulting in maximum research productivity as well as knowledge acquisition. I would also like to thank Dr. R.A. Merkel for providing knowledge and insight and for serving on my Master committee, James Liesman for providing statistical expertise, and Bob Patten for educational and interesting conversations. I thank the personnel at the Cytogenetics lab, especially Beverly Sankey and Joe Marrazza for their time and patience. I owe special thanks to Alan L. Grant for being a true friend and helping me each and every time I asked. I thank Dr. F.B. Livingstone for teaching me the importance of keeping an open mind and maintaining total objectivity. Special thanks are due Dr. Livingstone because he not only served as a committee member for my Master's degree, but also provided an extremely valuable link to the University of Michigan and all it's resources. Finally, I would like to show my appreciation for my parents who have provided support and respected decisions I have made throughout my college career.

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INTRODUCTION

Although the field of cytogenetics is a relatively young discipline, it has matured at an astounding rate. This branch of science concerns itself with the storage, duplication, transmission, and recombination of information necessary for the development and functioning of an organism. The quintessential component which allows these events to occur is deoxyribonucleic acid (DNA). In essence, the genetic material known as DNA is "the thread of life".

Genetics has, as do most sciences, a central theory. Bunge (1967) describes a theory as having three integral parts: the presence of a high level construct, the presence of a mechanism, and a capability for explanation. Vogel and Motulsky (1986) categorize the science of genetics with the statement, "In genetics, the high-level 'construct' is the gene as a unit of storage, transmission, and realization of information." Although our understanding has evolved, becoming more complex with the passage of time, the mechanisms have continuously been studied since the rediscovery of Mendel's laws in 1900. Other avenues of interest include deciphering the genetic code, determining protein function as coded for, and investigating transcriptional as well as translational systems. The

capability for understanding is near, but the explanation(s) are far from being understood (Bunge, 1967).

In humans, chromosomes have been meticulously characterized using a variety of well validated techniques in an attempt to more fully understand diseases and anomalies associated with chromosome aberrations. Detailed cytogenetic studies in domestic species are currently lacking. Furthermore, these studies generally have not reached the level of sophistication that is characteristic of human studies. It is for this reason that chromosomes of domestic animals are rarely observed in mid or late prophase. It is important to be able to isolate elongated chromosomes because chromosomes isolated in mid or late prophase provide much more information than chromosomes at metaphase. Techniques commonly used in human mid to late prophase chromosome preparations must be modified in order to characterize bovine chromosomes. Such modifications have not been thoroughly documented. Therefore, the objective of this study was to modify and validate a high-resolution G-banding technique to characterize bovine chromosomes.

REVIEW OF LITERATURE

History of Cytogenetics. The concept of genetics is very old. It is only logical to assume that even the earliest civilized man took note of the differences in appearance between animals and among creatures of the same type. One of the earliest recorded pieces of work which eluded to a genetic mode of inheritance can be found in text credited to Hippocrates. Hippocrates wrote:

> Of the semen, however, I assert that it is secreted by the whole body - by the solid as well as by the smooth parts, and by the entire humid matters of the body...The semen is produced by the whole body, healthy by healthy parts, sick by sick parts. Hence, when as a rule, baldheaded beget baldheaded, blue-eyed beget blue-eyed, and squinting, squinting; and when for other maladies, the same law prevails, what should hinder that longheaded are begotten by longheaded?

The Athenian philosopher Anaxagoras (500-428 B.C.) held similar beliefs. He wrote: "...in the same semen are contained hairs, nails, veins, arteries, tendons, and their bones, albeit invisible as their particles are so small. While growing, they gradually separate from each other." (from Vogel and Motulsky, 1986).

Aristotle put forth a comprehensive theory of inheritance. He believed in unequal contribution by the male as compared with the female parent. The male was said to contribute the impulse for movement while the female

provided the substance. Aristotle suggested that if the male impact was dominant, then a son was born, and if the female component was stronger, then a daughter was born. Plato, in his Statesman (Politikos), expanded the concept of inheritance with the suggestion that not only were the physical traits passed from parent to offspring, but so were the traits of personality. Plato believed that courageous produced courageous and inferior produced inferior. Democritus was in opposition to Plato's beliefs. He wrote: "More people become able by exercise than by their natural predisposition." (from Vogel and Motulsky, 1986). The disagreements between Plato and Democritus is indicative of the fact that the imminent nature-nurture problem had begun.

Mercado (1605), a Spanish physician, gave hints that the overwhelming influence of Aristotle, with regards to inheritance, was soon,if only gradually, to change. Mercado held that both the mother and the father contribute a seed to the subsequent child. Shortly thereafter, Malpighi (1628-1694) presented the hypothesis of "preformation", which suggests that the organism is preformed and complete in the ova, only to grow in size later. With the discovery of sperm by Leeuwenhoek, vanHam and Hartsoeker in 1677, the preformation theory was not completely abandoned. It was modified, and believed by some that the individual was "preformed" in the sperm, only to be nurtured in the ova (from Vogel and Motulsky, 1986). Factions of "ovists" and "spermists" struggled amongst one another until Wolff (1759)

declared that both sides were wrong and that empirical research was a necessity. Empirical research was carried out by Koelreuter (1733-1806) and Gaertner (1772-1850) on plant heredity. This was the ground work from which Mendel expounded (Vogel and Motulsky, 1986).

Joeseph Adams, a British physician, made an impact on the scientific community in 1814 with the publication of his book "A Treatise on the Supposed Hereditary Properties of Diseases" which was an attempt to provide a base for genetic counseling. It was an exceptional piece of work that made note of the following: a) offspring with recessive diseases frequently have related parents, 2) diseases that appear clinically identical may have different causes, and 3) new mutations sometimes occur where there is no family history at all (Motulsky, 1959). Next, came the work of F. Galton. Galton (1865) published a work entitled "Hereditary Talent and Character" which expressed the notion that through controlled breeding, virtually any characteristic desired could be achieved in animals. This included physical traits as well as mental. Galton believed animals as well as humans were as pliable as plastic and that eugenics were the means to justify the end; the end was an improved human species (Galton, 1865).

On February 8 and March 8, 1865, Gregor Mendel read his work entitled "Experiments in Plant Hybridization" at the Natural Science Association in Brun, Czechoslovakia. Mendel looked at the assortment of specific traits in peas and

subsequently developed the laws of segregation. This was the birth of the "gene" concept for the transmission of hereditary information (from Peters, 1959).

Robert Hooke, while examining thin slices of cork in 1665, identified "cells" as the structural unit in plants (from Bradbury, 1968). From this time to Nageli's discovery in 1842, cytogenetics experienced little if any evolution. Nageli observed structures which were visible only in some cells and only at certain times. The term Nageli used to describe these structures was "transitory cytoblast". These structures were later named chromosomes by Waldeyer in 1888 (chromo- meaning stained and -some meaning body). After Nageli's discovery, the evolution of cytogenetics was extremely rapid. In 1875, Hertwig was the first to actually observe the process of animal fertilization and the presence of a nucleus with continuity. Flemming (1880-1882) observed the separation of sister chromatids during mitosis and van Beneden noted the equal distribution of chromosomes among daughter cells. In 1885, Nageli put forth the concept of "idioplasma" which referred to the plasma portion of the cell that contained hereditary information (from Vogel and Motulsky, 1986). Rabl in 1885 suggested a model which predicted that chromosomes maintain an anaphase-like conformation and remain in distinct domains during the cell cycle. Rabl observed chromosomes as being lined up in the nucleus with centromeres towards the interior and chromosome arms parallel and attached to the opposite side of the

nucleus (from Saumweber, 1987). Boveri (1888) found that chromosomes were paired and that each pair had unique morphological characteristics. Stern (1959) indicated that the study of human cytogenetics began with work by Arnold (1879) and Flemming (1882) who both studied human mitotic chromosomes. These studies led to the chromosomal theory of Mendelian inheritance by Sutton and Boveri in 1902. Subsequently, McClung (1902) and Wilson (1905) discovered that the determination of sex is controlled at the chromosomal level (Eldridge, 1985), thus providing some of the first work supporting the idea that chromosomes carry hereditary information. Shortly thereafter, in 1909 the Danish geneticist Johannsen described the Mendelian units of heredity as "genes" (from Peters, 1959). Disturbances in chromosomal distribution were first reported by Bridges (1916) and were referred to as nondisjunction which was later found to be the cause of Down's Syndrome (Lejeune, 1959).

The first observance of human mitotic chromosomes is believed to have been by Arnold (1879). The first attempt at determining the number of chromosomes in the human was by von Winiwarter (1912). He took testicular samples from four men aged 21, 23, 25, and 41. In addition, he obtained three oogonial mitoses from a four month old female. Winiwarter concluded that males have 47 chromosomes, and that females have 48 chromosomes. He observed the sex chromosomes in the male, but interpreted them as being a single entity.

Painter, in 1921 and 1923 examined testicular samples from three individuals from the Texas State Insane Asylum (Painter, 1923). In the preliminary report of 1921, Painter described the male as having either 46 or 48 chromosomes,but in the final report of 1923, he settled upon 48 as the correct number of chromosomes due probably to fear of persecution from the scientific community. In addition, Painter was the first to demonstrate a sex bivalent in chromosome morphology consisting of the X and Y chromosomes which migrated to opposite poles in anaphase. Painter's estimation of 48 chromosomes became so deeply etched in investigators minds that more than 30 years later, when improved techniques for counting were available, it was reported that there were 48 chromosomes in the human (Hsu, 1952). In 1955, Tjio and a Swedish cytogeneticist, Levan, improved upon chromosomal preparation techniques with the advent of short hypotonic exposure times and the addition of colchicine to the preparation (Tjio and Levan, 1956). Lung fibroblasts from four human embryos were examined, and only 46 chromosomes could be found. Levan and Tjio (1956) were careful in the report of their findings. They suggested that a 2n=46 could be a viable explanation. Ford and Hamerton (1956) provided support for Levan and Tjio's findings when they published their findings of 46 chromosomes in male testicular tissue. The birth of clinical cytogenetics occurred three years after the acceptance that 46 chromosomes was the correct number for

both the male as well as the female human. The year 1959 is a well published year for chromosomal abnormalities. Lejeune et al. (1959) described the cause of Down's Syndrome as being the result of having 47 chromosomes, i.e., trisomy 21. Jacobs and Strong (1959) noted that individuals exhibiting symptoms of Klinefelter's Syndrome also possessed 47 chromosomes. The supernumerary chromosome was subsequently identified as an X, hence the karyotype was designated as 47 XXY. Another discovery in 1959 was by Ford et al. (1959). They described a case of Turner's Syndrome in which a woman with atypical physical features possessed a karyotype of only 45 chromosomes. An X chromosome was absent and the karyotype was designated 45 XO. A fourth chromosomal numerical aberration was noted by Jacobs et al. (1959b) in which a woman with slight mental retardation accompanied with dysfunction of the sexual organs was found to have 47 chromosomes, three of which were X's.

Amethopterin - attainment of elongated chromosomes. The study of chromosomes has advanced over the last several years predominately as a result of the application of banding techniques on metaphase chromosomes (Yunis and Chandler, 1977). Attempts to analyze chromosomes at an earlier stage in cellular division (mid to late prophase) have been made in order to gain additional information which can be acquired from the more finely banded chromosome (Prieur et al., 1973; Skovby, 1975; Bigger and Savage, 1975; Yunis and Sanchez, 1975). These early attempts have resulted in limited success due to the low number of early mitotic cells obtained when using standard culture techniques. No agent has been found which selectively arrests a mitotic cell at prophase, a stage at which the chromosomes are less tightly condensed (Harnden et al., 1981). Yunis (1976) made a dramatic impact on the field of cytogenetics when he combined a direct Wright staining method with a low colcemid exposure and a synchronization of the cell cycle with amethopterin. Amethopterin addition to the cell culture system prevents cells from replicating their DNA, thereby causing a large number of cells to accumulate at the G1/S border of the cell cycle. The addition of thymidine to the culture will cause a subsequent release of the amethopterin imposed block. The cells will now proceed to complete DNA replication and then continue to advance through mitosis in a wave which can be stopped at a precise time with the addition of colcemid (Yunis et al.,

1978).

Amethopterin, also known as methotrexate, has for some time been known to be a folate antagonist which has a potent inhibitory effect on the enzyme dihydrofolate reductase (Werkheiser, 1961; Bertino, 1963; Werkeiser, 1963). This class of folic acid analog was the first antimetabolite to demonstrate profound though temporary remission in leukemia (Farber et al., 1948). Subsequently, it was demonstrated to dramatically resolve uterine trophoblastic tumors (Li et al., 1956). Amethopterin was also the first drug to provide a cure for choriocarcinoma in women (Hertz, 1963). It was this attainment of a high percentage of permanent remissions in this otherwise lethal disease that provided great impetus for the investigation of chemotherapeutic compounds (Huffman et al., 1973). Since amethopterin has been established as a tool in chemotherapy, significant antitumor activity has also been observed in patients with carcinoma of the head and neck (Capizzi et al., 1970; Mitchell et al., 1968). In addition, when treated with amethopterin, nonneoplastic disorders such as psoriasis (McDonald et al., 1969; Van Scott et al., 1964), Wegener's granulomatosis (Von Leden, 1964), saroidosis (Lacher, 1968), corticosteroid dependent asthma (Mullarkey et al., 1988) and rheumatoid arthritis (Groff et al., 1983; Williams et al., 1985) show diminished severity of symptoms.

Amethopterin is an antimetabolite with a molecular weight of 454.46 and is chemically described as N-[p-[[(2,4-

diamino-6-pteridinyl)methyl]-methylamino]benzoyl]glutamic acid. The principle mechanism of action of amethopterin is the competitive inhibition of the enzyme folic acid reductase. For the processes of DNA synthesis and cellular replication to occur, folic acid must be reduced to tetrahydrofolic acid by this enzyme. Folic acid is an extremely polar molecule that requires specific transport mechanisms to enter mammalian cells. Upon entry into the cell, the enzyme folylpolyglutamate synthetase adds an additional glutamyl residue to the molecule. When methotrexate is present, it enters cells by means of an energy-dependent, temperature-sensitive, concentration-dependent process (Goldman et al., 1968) involving a specific intramembrane protein (McHughes and Cheng, 1979). This influx process has been observed in isolated membrane vesicles (Young et al., 1979). Once inside the cell the methotrexate is transformed into a polyglutamated form (Jolivet and Schilsky, 1981). These methotrexate polyglutamates are poor or incapable at crossing a cell membrane to the extracellular region (Sirotnak et al., 1978). Intracellular methotrexate polyglutamates with as many as five glutamyl residues have been identified; thus the methotrexate causes entrapment of the glutamyl residues, thereby preventing their addition to the folate. This entrapment is important because evidence indicates that polyglutamylated folates have a substantially greater affinity than the monoglutamylated folates for enzymes such

as thymidylate synthetase (Gilman, et al., 1985). Folate must first be reduced by dihydrofolatereductase (DHFR) to tetrahydrofolate (FH_4) to serve as a cofactor in one-carbon transfer reactions. Single carbon fragments are enzymatically added in various configurations to FH_4, after which time they may be transferred in specific synthetic reactions (Gready,1979). Conversion of 2-deoxyuridylate (dUMP) to thymidylate, an indispensable component of DNA, is catalyzed by thymidylate synthetase (Tattersall et al., 1974; Grafstrom et al., 1978). The methyl group is transferred from N^{5-10} -methylene FH_4 to the uracil moiety of dUMP, specifically to the pyrimidine ring at the oxidation level of formaldehyde. The formaldehyde is subsequently reduced to methyl by the pteridine ring of the folate coenzyme resulting in the formation of dihydrofolate (FH_2). To function again as a cofactor, the FH_2 must be reduced to FH_4 by DHFR. Inhibitors which possess a high affinity for DHFR prevent the formation of FH_4 and cause major disruptions in normal cellular metabolism by producing an acute intracellular deficiency of folate coenzymes. The folate coenzymes cannot function metabolically because they become trapped as FH_2 polyglutamates. A repercussion of this entrapment is that one-carbon transfer reactions crucial for the de novo synthesis of purine nucleotides and of thymidylate cease; there is a subsequent interruption of the synthesis of DNA and RNA as well as other vital metabolic reactions.

An understanding of the above events enables an appreciation of the rationale for using 5-methyltetrahydrofolate, thymidine and/or leucovorin (5-formyltetrahydrofolate) in the "rescue" of cells from toxicity induced by drugs such as amethopterin (Pinedo et al., 1976). Leucovorin enters cells via a specific carrier-mediated transport system. It is a fully reduced, metabolically functional folate coenzyme and is convertible to other folate cofactors. Because of these characteristics, it may function directly, without the need for reduction by DHFR in reactions such as those essential for purine biosynthesis. Alternatively, the addition of thymidine may also rescue a cell. Thymidine may be converted directly to thymidylate by thymidine kinase, thus bypassing the reaction catalyzed by thymidylate synthetase and as such provide the necessary precursor for DNA synthesis (Jackson, 1980).

An important feature of the binding of active folate antagonists with DHFR is the extremely low inhibition constant which has been observed to be on the order of 1 nM. Despite the exceptionally high affinity of the antagonists for the DHFR protein molecule, covalent bonds are not involved in the enzyme/inhibitor interactions (Matthews et al., 1978; Chabner, 1982).

As is the case with most inhibitors of cellular reproduction, amethopterin exhibits a selective effect which is obtainable to only a partial extent. This is true

because folate antagonists kill cells which are in the S phase of the cell cycle. In addition, evidence indicates that methotrexate is substantially more effective when the cellular population is in the logarithmic phase of growth as opposed to being in the plateau phase. Methotrexate is also capable of inhibiting RNA and protein synthesis resulting in the slowing of cells entering into S phase; its cytotoxic actions are said to be "self-limiting" (Skipper and Schabel, 1982).

Cell synchronization with a methotrexate induced block and subsequent release from inhibition by the addition of thymidine, can be of great importance in the cytogenetic studies of cell cultures (Yunis et al., 1978). The advantages of cell synchronization are two-fold: 1) mitotic index is improved for most cell cultures and 2) the harvest of chromosomes can be optimized in such a way as to capture cells closer to prophase than metaphase, resulting in greatly elongated chromosomes (Yunis, 1981). Such preparations may dramatically increase the resolution of banding that can be achieved. Although considerably more information is available in cells caught at or near prophase, accompanying this increase in information is a significant increase in the complexity of analysis. To date, the majority of the work on high-resolution banding has been performed on human peripheral blood lymphocytes. The intricacies of cellular division kinetics in this system have been thoroughly studied.

Phytohemagglutin: Stimulation of lymphocyte proliferation. In principle, chromosome preparations may be made from any tissue or suspension that contains mitoses. The most convenient technique is blood culture due to the ease of obtainment. The blood of healthy, nonleukemic individuals contains no actively dividing cells. Therefore, cellular division must be induced artificially through the use of a mitogen. Most mitogenic plant lectins stimulate only thymus dependent lymphocytes (T-cells) and are inactive for mitosis of thymus independent lymphocytes (B-cells) (Waxdal, 1978). Phytohemagglutinin is a example of such a mitogen and was the mitogen of choice for this experiment. Lymphocytes are stimulated to enter a metabolically more active (25 to 50 fold greater than the rate of resting lymphocytes) state leading to the expression of a cell specific genetic program and finally, to DNA replication and mitosis (Kornfeld et al., 1972). One hour after PHA (phytohemagglutinin) incubation of a blood sample, T-cells begin to synthesize RNA and approximately 24 h later DNA synthesis follows (Hauser et al., 1976).

Upon completion of incubation, chromosome preparations are made. The nomenclature for high-resolution preparations of prophase and pre-metaphase human chromosomes has been described in detail (Karger et al., 1985). A similar system should be adapted for chromosomes from other species.

<u>Chromosome Banding</u>. The field of cytogenetics began with the discovery of chromosomes. An important need was a technique to characterize individual chromosomes. This was initially accomplished through determination of the characteristic number of chromosomes and description of the morphology of chromosomes for a given species. Shape and size of each chromosome at anaphase during meiosis or mitosis were the initial variables used to identify individual chromosomes. Location of primary constrictions (centromeres) was a determinant of chromosome shape. Positive identification of chromosomes on the basis of size and shape was inadequate. A new thrust to the field of cytogenetics was provided by Caspersson et al. (1970), who described a technique for banding human chromosomes. Since 1970, the field of cytogenetics has expanded dramatically as formerly undetected structural abnormalities have been revealed. Each chromosome can now be described by its characteristic banding pattern which is consistent under normal conditions across all cell types of a given individual.

A variety of staining procedures have become available since those of Caspersson et al. (1970). In the following section, major chromosomal banding techniques shall be addressed. These techniques include: Quinacrine or Q-banding, Giemsa or G-banding, Centromeric or C-banding, reverse Giemsa or R-banding, Telometric or T-banding, Nucleolar organizing region or NOR staining, Sister

chromatid exchange or SCE staining, early and late replicating banding, lateral asymmetry staining, conventional staining, and autoradiography. See Figure 1 for a diagrammatic representation of a hypothetical bovine chromosome that had been exposed to each of the previously described chromosome banding techniques. Bos taurus and Bos indicus each have 60 chromosomes in the diploid cell. All of the chromosomes except the sex chromosomes are acrocentric. The X chromosome is metacentric in both species whereas the Y chromosome is submetacentric in Bos taurus but acrocentric in Bos indicus.

Chromosome Banding Techniques

I) Conventional Staining Conventional staining of chromosomes is the simplest staining method. The most common type of conventional stain is a standard Giemsa solution, although 2% acetic orcein or 2% karmin solution will yield similar results. These dyes will intensely and uniformly stain the entire chromosome. Conventional staining technique may be of considerable use in general descriptive studies of chromosomes obtained from cell cultures. For diagnostic purposes such as screening for double minutes (miniature chromosome like structures often composed of duplications of genomic DNA) or diagnosis of common numeric aberrations, this method is adequate. To obtain a more detailed picture of chromosome structure or to identify

unequivocally individual chromosomes or chromosome fragments, banding methods need to be employed.

II) Giemsa (G-) Banding. The first to publish results on banded chromosomes obtained from staining chromosomes that had undergone a pretreatment enzyme digestion was Dutrillaux et al. (1971) although procedures were also published by Seabright (1971), Sumner et al. (1971) and Evans (1973). Evans' (1973) initial work had been done in 1971. Each of the above resulted in chromosomes with the same banding pattern. This banding is referred to as G-banding because it was Giemsa stain that was initially used after pretreatment of the chromosome with digestive enzymes. Digestive enzymes employed in chromosome banding studies included trypsin, chymotrypsin, pronase, and papain. Yunis (1977) described a number of pretreatment techniques which yield G-banded chromosomes.

G-bands may be obtained with the application of Romanovsky stains - Giemsa, Wright's, Leishman's and May-Grunwald's. G-bands are revealed by the application of various additional techniques which allow only the most readily staining chromosome segments to take up the dye. Advantages of this method over Q-banding or R-banding (discussed later) include the use of bright-field (as opposed to fluorescent) microscopy, and a permanent stain of the chromosome (fluorescent stains fade away).

III) Quinacrine (Q-) Banding. Q-banding makes use of DNA staining fluorochrome quinacrine mustard or quinacrine dihydrochloride, both acridine derivatives which stain mitotic chromosomes to produce a distinct pattern of cross-striations extending across both sister chromatids (Caspersson et al., 1968). These Q-bands occur on the same segments of the chromosome as G-bands and permit fluorimetric differentiations of all chromosomes (Caspersson et al., 1971, 1972). Pearson et al. (1970) made a significant discovery; while using quinacrine hydrochloride they were able to demonstrate the ability to sex human chromatin based on the premise that Y chromatin material would fluoresce. Hence the presence of a single fluorescent body indicated that the cell was male.

IV) Reverse (R-) Banding. Dutrillaux and Lejeune (1971) introduced reverse Giemsa or R-banding technique to cytogenetics. R-banding provides a reverse image behaving like a photographic negative of the image produced from G- or Q-bands (Yunis, 1977). Specifically, the band pattern along the chromosome is opposite in staining intensity to those produced by G- or Q-banding. The regions which have light Giemsa stain or weak fluorescence ,respectively, stain dark by R-banding (and vice versa). R-bands are stained after controlled heat or pH denaturation (Schested, 1974). Q-bands indicate A-T (adenine-thymine) rich chromosome segments while R-bands indicate G-C (guanine-cytosine) rich

segments which possess a greater resistance to heat denaturation than do the A-T abundant regions (Dutrillaux and Lejeune, 1975).

A wide variety of stains (some fluorescent and others not) may be used to produce reverse-banded chromosomes, when the appropriate treatments are applied. These stains include Giemsa, Acridine Orange, Chromomycin, olivomycin, DAPI, and metramycin (Verma et al., 1977; Schweizer, 1976).

V) Telomeric (T-) Banding. T-banding is an offshoot of R-banding involving staining telomeres of chromosomes. T-banding is accomplished by applying treatments used in R-banding with the only exception being an increase in severity, i.e., increasing the high temperature incubation time or by lowering pH of buffer (Yunis, 1977). This technique is useful because it affords more precise banding of the terminal regions of chromatids. These regions often are not clear when other techniques are used.

VI) Constitutive Heterochromatin (C-) Banding. C-banding specifically stains areas of constitutive heterochromatin which is most commonly found in, and immediately adjacent to, the centromere. C-banding was first discovered by Pardue and Gall in 1970 while performing a radioactive nucleic acid hybridization experiment. They noted the appearance of densely stained centromeric heterochromatin that appeared when NaOH treated chromosomes

were subsequently stained with Giemsa. While C-bands occur in all mammalian species, amount and distribution between species may vary considerably. Regions stained using this technique are located around centromeres, on short arms or satellite regions of acrocentric chromosomes, at secondary constriction regions of chromosomes and at the distal end of the Y chromosome. These species differences differences may provide an effective cytogenetic marker for species identification (Pathak and Hsu, 1985).

VII) Silver Staining of Nucleolus Organizer Regions (NOR-) Banding. Matsui and Sasaki (1973) discovered a technique which resulted in an ordered precipitation of silver granules on chromosomes in a species specific pattern. Staining of NORs reveals transcriptionally active ribosomal cistrons in mammalian cells. Only nucleolus organizers which are functionally active in the previous interphase are stained (Schwarzacher and Wachtler, 1983).

An illustration of the various stains and subsequent banding patterns is shown in Figure 1.

TYPICAL BANDING PATTERNS OF A BOVINE CHROMOSOME

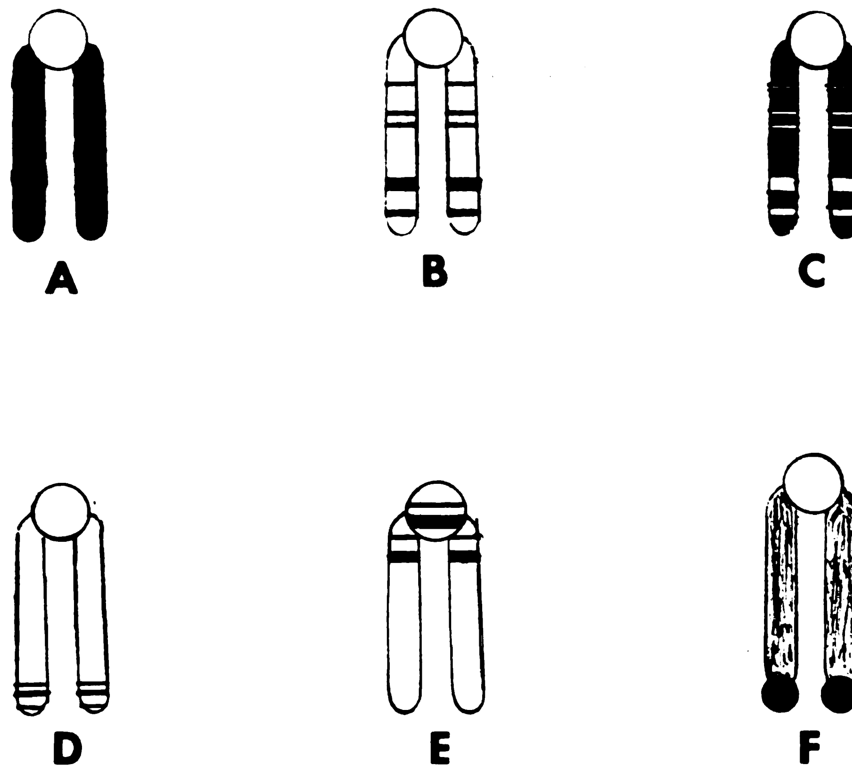


Figure 1. Techniques illustrated are A) Conventional, B) Giemsa and Quinacrine, C) Reverse, D) Telomeric, E) Constitutive Heterochromatin, F) Silver Staining of Nucleolus Organizer Regions.

MATERIALS AND METHODS

The purpose of this experiment was to follow a validated procedure used to obtain elongated (mid to late prophase) chromosomes in humans and adapt it for use in the bovine species, Bos taurus.

Growth of Cells. The following procedures are modifications of Yunis et al. (1978). Heparinized peripheral blood (0.3 ml) was cultured in 5 ml MEM (minimum essential media - GIBCO, Grand Island Biological Co., Grand Island, NY) supplemented with glutamine (10^{-5} M), 10% gamma ray irradiated fetal calf serum (Sigma Chemical Company, St. Louis, MO), 50 ug gentamicin/ml media (GIBCO) and 0.2 ml phytohemagglutinin M (GIBCO) in 15 ml sterile disposable, conical centrifuge tubes (Corning Glassware, Corning, N.Y.). Cultures were incubated for 72 h at 39° C. Amethopterin (methotrexate; Lederle Laboratories, Pearl River, NY) at a final concentration of 10^{-7} M was then added to induce synchrony. After 16, 16.5 or 17 h, cells were released from the amethopterin block by two washes with unsupplemented media. The cells were then resuspended in supplemented media containing 10^{-5} M thymidine (Sigma) and incubated again at 39° C. Each amethopterin treatment (16, 16.5, 17 h

exposure time) was divided into 9 thymidine treatment groups. Thymidine exposure time varied from 4 to 6 h in 15 minute increments. Time was measured from addition of thymidine to the addition of colcemid (GIBCO), a drug which disrupts the spindle apparatus. Cells were exposed to colcemid (GIBCO) at 0.06 ug/ml for 10 min.

Chromosome Harvest. In order to obtain acceptable preparations, it was critical that careful harvesting techniques be employed. Following colcemid exposure for 10 minutes, cells were immediately centrifuged at 275 X g for 10 min. The supernatant was drawn off to just above the pellet. The pellet was agitated gently and resuspended in 0.075 M KCl at 39°C, and incubated at 39°C for 10 min. The suspension was centrifuged for 10 minutes at 275 X g and then the supernatant was again removed to just above the pellet. The pellet was agitated gently and resuspended in 6 ml fresh 3:1 absolute methanol:glacial acetic acid fixative. After 10 minutes of fixation, the cells were again centrifuged for 10 minutes at 275 X g. The supernatant was removed and 6 ml fresh fixative were added. The above fixation procedure was repeated for a total of three to six times to eliminate cellular debris and ensure excellent spreading and staining of chromosomes during mitoses.

Slide Preparations. Slide preparations were made by drawing off all but about 0.5 ml of the fixative without

disturbing the pellet, then gently aspirating with a pipette to resuspend the cells. A small amount of the suspension was drawn into a Pasteur pipette and then dropped onto a slide held at a 30 to 45 degree angle from a height of 70 to 90 cm. The slides had previously been cleansed in 95% ETOH and rinsed in distilled water. The slide was blown on (by investigator) to aid in spreading the droplets. The slides were then placed on a 55° C hot plate until dry. Slides were examined under a phase contrast microscope at 100 X magnification to determine the presence or absence of mitoses. If no chromosome spreads were located, then the slide was not further processed. If a chromosome spread was located, then the slide was aged for at least 24 h at room temperature, or for 45 minutes to 1 h in an oven at 95° C if staining was performed the same day as harvest.

Staining. The next step was the trypsin G-banding staining procedure (modified from Sanchez et al., 1973). Five ml of 0.25% trypsin (GIBCO, 1x) was combined with 50 ml of phosphate buffered saline (8.0 g NaCl, 0.2 g KCl, 1.5 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.01 g phenol red). The trypsin solution was poured into a coplin jar. Two additional coplin jars were filled with a 0.9% NaCl solution (for rinsing slides after trypsin treatment).

Slides were submerged in trypsin for 5 to 25 sec, submersion time was determined by viewing a test slide under a 1000 X magnification microscope. Under-trypsinized

chromosomes had thin, dark sister chromatids which were widely separated. Over-trypsinized chromosomes appeared fuzzy and the bands frayed out with sister chromatids that were thick and lacked boundaries. After each trypsin treatment, trypsin was rinsed off by sequentially dipping the slide into the two NaCl solutions. Excess fluid was shaken off. Immediately prior to staining, 1 ml stain solution (for formulation see Appendix 1) was combined with 3 ml of Gurr's buffer (pH 6.8) in a small vessel. The stain-buffer solution was poured onto a horizontally situated slide. After 20 to 90 sec (correct time was determined by viewing chromosomes at 1000 X magnification) stain was removed from the slide by liberally rinsing with distilled water which was applied with a wash bottle. The slide was dried by placing in front of an air jet, and then a cover slip was applied using Permount mounting media. Once Permount had dried, the slide was complete and ready for viewing.

Scanning For Chromosomes. The finished slide was placed on the microscope stage. Three complete longitudinal scans were made starting at the leading edge of the slide end. The first scan was at a distance 25% into the interior of the slide, the second scan bisected the slide, and the third scan began 25% of the distance from the rear or trailing edge. Scanning was carried out at 100 X magnification. When a chromosome spread was located, the microscope was

changed to 1000 X magnification oil immersion objective. The chromosome position was recorded and the chromosome stage was determined at this time or photographed with Kodak 160 ISO Techpan film for subsequent analysis.

Because of the broad nature of the experimental design, there were treatment groups that resulted in zero or a very low number of chromosome spreads being located during the three scans of the slide. It was arbitrarily decided that any slide possessing less than 10 chromosome spreads was not considered in the determination of the optimum treatment combination for obtainment of chromosomes with a haploid total band number of greater than 310. A chromosome 'spread' is defined as a diploid set of chromosomes containing the full compliment of 60 chromosomes when viewed at 1000 X magnification. Chromosome spreads with greater than 310 bands were recorded as "positive" and chromosome spreads of less than 310 bands were recorded as negative. High-resolution karyotypes are generated exclusively from the positive group of chromosomes. The internationally accepted G-banded karyotype standard for cattle contains 310 bands per haploid set of chromosomes which are believed to be at the mid-metaphase stage (Ford et al., 1980). The number of positive and negative spreads were also expressed as a percent of total spreads.

Statistical Analysis. Data were statistically analyzed using the Statistical Analysis System (SAS, 1988). Least

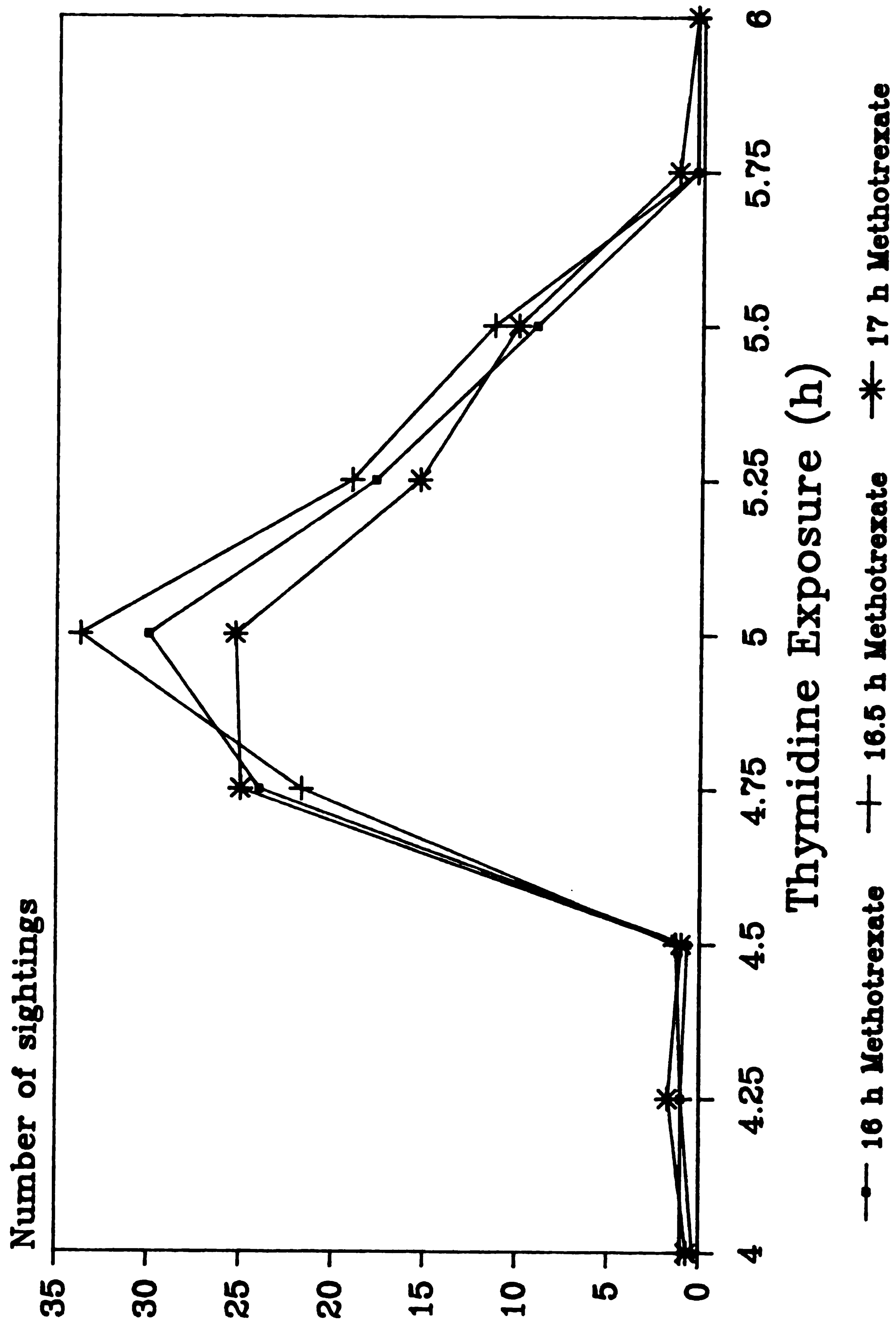
square means were generated with the General Linear Model of SAS. Analysis of variance was conducted with methotrexate and thymidine exposure times serving as main effects. Determination of homogeneous variance was accomplished with the use of Bartlett's test (Gill, 1978).

RESULTS AND DISCUSSION

Preliminary experiments were performed to determine an appropriate dose of methotrexate and a suitable growth medium for bovine lymphocytes. Methotrexate was found to elicit an effect as a mitotic inhibitor at a final concentration of 10^{-7} M when cells were exposed for 16.5 h. A medium found to support a high level of bovine lymphocyte proliferation was MEM (minimum essential media - GIBCO) with supplements (PHA, serum, glutamine).

Figure 2 is a histogram of the total number of chromosome spreads sighted for each treatment group regardless of usability. In treatment groups that were exposed to thymidine for 4 to 4.5 and after 5.75 h, the number of chromosome spreads sighted was less than the limit stated in Materials and Methods and thus was not included in subsequent analysis. Figure 2 shows the average number of chromosome spread sightings in three methotrexate treatment groups with each methotrexate group being subdivided into 9 thymidine time exposures. The majority of all chromosome observations occured between 4.5 and 5.75 h of thymidine exposure. The time a cell spends in mitosis is approximately 1 h which corresponds to the frequency profile generated in Figure 2.

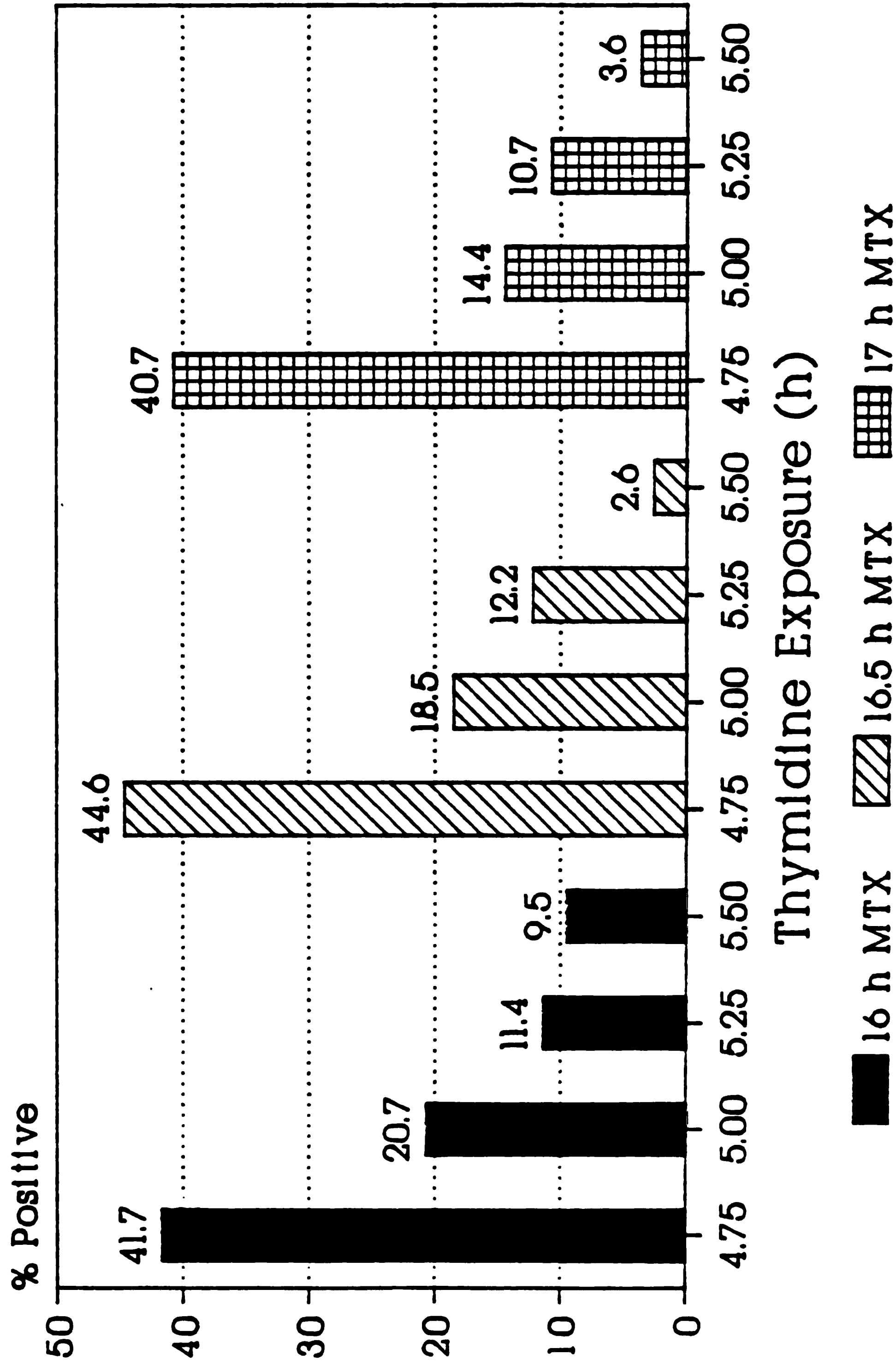
Figure 2. Average number of chromosome sightings for three methotrexate time exposures (16, 16.5, and 17 h), each of which included nine thymidine time exposures (4, 4.25, 4.5, 4.75, 5, 5.25, 5.5, 5.75, and 6 h)



Number of sightings
35
30
25
20
15
10
5
0
4
4.25
4.5
4.75
5
5.25
5.5
5.75
6
Thymidine Exposure (h)
16 h Methotrexate
16.5 h Methotrexate
17 h Methotrexate

Figure 3 is a histogram of thymidine exposure time plotted against the percent positive chromosome spreads in each of three MTX (methotrexate) groups at various exposure times. There was a consistent decrease in percent positive chromosome spreads for each MTX group with increasing thymidine exposure time from 4.75 to 5.5 h. The greatest percent of positive chromosomes was obtained at 4.75 h of thymidine exposure for all methotrexate exposure times. Percent positive chromosomes obtained at 4.75 h of thymidine exposure and 16.5 h of methotrexate exposure was slightly greater than any other treatment combination considered. This treatment combination was optimum under conditions of this experiment. The trend depicted in Figure 3 is consistent for each of the three methotrexate treatment groups and occurs because a given cell at a given time is proceeding through cellular division. Methotrexate is an inhibitor of cellular division that elicits its effect during the S phase of the cell cycle (Hirsch et al., 1987). Upon application of excess thymidine, the arrested cell is released from inhibition and again proceeds toward division. Due to the synchronized fashion in which cells approach division, it would be expected that few cells would be found in mitosis at a time earlier than the genetically determined time as measured from S phase to mitosis. It is also likely that a cell found in the thymidine exposure groups of 4.0, 4.25, or 4.5 h is a cell that has not been effectively inhibited by methotrexate. A decline in the percent

Figure 3. Number of chromosome spreads with greater than 310 bands expressed as a percent of total spreads observed (% positive) at three methotrexate (MTX) exposure times (16, 16.5, and 17 h) each of which included four thymidine exposure times (4.75, 5, 5.25, and 5.5 h).


% Positive
50
40
30
20
10
0
41.7
20.7
11.4
9.5
44.6
18.5
12.2
2.6
40.7
14.4
10.7
3.6
4.75
5.00
5.25
5.50
4.75
5.00
5.25
5.50
4.75
5.00
5.25
5.50
Thymidine Exposure (h)
16 h MTX
16.5 h MTX
17 h MTX

positive chromosome spreads after the synchronized wave of cells arrive at mitosis results from cells proceeding through mitosis. The reason for this decline in percent positive chromosome spreads may be that chromosome length decreases as a cell advances toward metaphase from prophase. An explanation for the observed decline in frequency of chromosome observations is that mitosis has a duration of approximately 1 h and hence after actual division the cell enters interphase of the cell-cycle, not to divide again for several hours (Inoue, 1981).

Analyses of different stages of chromosome condensation were made as a function of thymidine and methotrexate exposure duration. Table 1 shows the effects of various exposure times of methotrexate and thymidine on the percent positive chromosome spreads. From the results given in Table 1, the combination of treatments to yield the greatest fraction of positive chromosomes appears to be a 16.5 h methotrexate exposure accompanied by a 4.75 h thymidine exposure.

Means and standard deviations for the number of positive, negative, and total chromosome spreads in each treatment group are reported in Table 2. The greatest number of positive chromosome observations was at 4.75 h in each methotrexate treatment time. The greatest total number of observations was found at 16.5 h methotrexate, 5 h thymidine exposure. Two reasons may account for this observation 1) fewer cells were synchronized at methotrexate

Table 1. Means and standard deviations (SD) for percent positive chromosome spreads at various MTX and THY exposure times[1]

			% Positive[2]	
MTX	THY	N	Mean	SD
16.00	4.75	3	41.7	3.0
16.00	5.00	3	20.7	3.5
16.00	5.25	3	11.4	1.0
16.00	5.50	2	9.5	0.6
16.50	4.75	3	44.6	0.7
16.50	5.00	3	18.5	4.0
16.50	5.25	3	12.2	1.9
16.50	5.50	3	2.6	4.4
17.00	4.75	3	40.7	3.0
17.00	5.00	3	14.4	1.5
17.00	5.25	3	10.7	1.4
17.00	5.50	3	3.6	5.1

[1]MTX - Methotrexate exposure (h)
THY - Thymidine exposure (h)

[2]Different among MTX exposure times ($P < .031$); different among THY exposure times ($P < .0001$); MTX * THY interaction ($P < .1261$)

Table 2. Means and standard deviations (SD) for the number of positive, negative, and total chromosome spreads in each treatment group[1].

			Positive[2]		Negative[3]		Total[4]	
MTX	THY	N	Mean	SD	Mean	SD	Mean	SD
16.00	4.75	3	10.0	2.0	14.0	2.6	24.0	4.4
16.00	5.00	3	6.3	2.1	23.7	3.5	30.0	5.6
16.00	5.25	3	2.0	0.0	15.7	1.5	17.7	1.5
16.00	5.50	2	1.0	0.0	9.5	0.7	10.5	0.7
16.50	4.75	3	9.7	1.5	12.0	2.0	21.7	3.5
16.50	5.00	3	6.3	2.3	27.3	2.5	33.7	4.7
16.50	5.25	3	2.3	0.6	16.7	1.5	19.0	2.0
16.50	5.50	3	0.3	0.6	11.0	1.0	11.3	1.5
17.00	4.75	3	10.3	4.2	14.7	4.0	25.0	8.2
17.00	5.00	3	3.7	0.6	21.7	2.1	25.3	2.5
17.00	5.25	3	1.7	0.6	13.7	3.2	15.3	3.8
17.00	5.50	3	0.5	0.7	12.5	0.7	13.0	1.4

[1]MTX - Methotrexate exposure (h)
THY - Thymidine exposure (h)

[2]Different among MTX exposure times ($P < .563$); different among THY exposure times ($P < .0001$); MTX * THY interaction ($P < .708$).

[3]Different among MTX exposure times ($P < .486$); different among THY exposure times ($P < .0001$); MTX *THY interaction ($P < .106$).

[4]Different among MTX exposure times ($P < .600$); different among THY exposure times ($P < .001$); MTX * THY interaction ($P < .307$).

exposure of less than 16.5 h, and 2) cell demise occured at 17.0 h MTX exposure.

Length of thymidine exposure significantly affected number of positive spreads ($P < 0.0001$) for all treatment groups (Tables 1 and 2). Thymidine demonstrated a substantial effect on not only number, but also length of individual chromosomes. This is expected since thymidine permits a cell arrested at S phase by MTX to commence cellular division again. Progression of mitosis past the prophase stage results in the continuous reduction of chromosome length. Methotrexate exposure time did not significantly affect the absolute numbers of chromosome sightings (Table 2; $P > .10$). However, MTX exposure times appeared to significantly affect sightings of metaphase chromosome spreads when data was expressed on a percent basis (Table 1; $P < .031$), but this was a statistical artifact resulting from percent values having non-linear functionality. As calculated using Bartlett's statistical test for variance in a data set (Gill, 1978), there was homogeneous variance ($P > .25$) for the percent positive chromosome spreads.

The optimum treatment combination for the isolation of elongated _Bos taurus_ chromosomes was 16.5 h MTX exposure followed by 4.75 h of thymidine exposure. Yunis et al. (1978) reported optimum thymidine exposure time to be from 5 h 5 min to 5 h 10 min in humans. Ianuzzi et al. (1987) found optimum time to be 5.5 to 6.0 h in cattle. In

contrast, DiBerardino et al. (1985) found optimum times to be 4 to 5 h. Differences in optimum thymidine exposure times may be due to variations in techniques, among species, and within species.

Figures 4, 5, 6, and 7 are actual photographs of mitoses as they appear when viewed through a microscope at 1000 X magnification. Chromosome haploid set band numbers of approximately 200, 250, 300, and 500, respectively, are shown. Figure 6 typifies a mid-metaphase mitoses and is borderline to being considered high-resolution whereas Figure 7 is an example of a late prophase mitoses that is clearly high-resolution. Figures 4 and 5 are not considered as high-resolution (high-resolution: greater than 310 bands per haploid chromosome set).

Figure 4. A _Bos taurus_ mitoses of approximately 200 bands per haploid set of chromosomes.

Figure 5. A _Bos taurus_ mitoses of approximately 250 bands per haploid set of chromosomes.

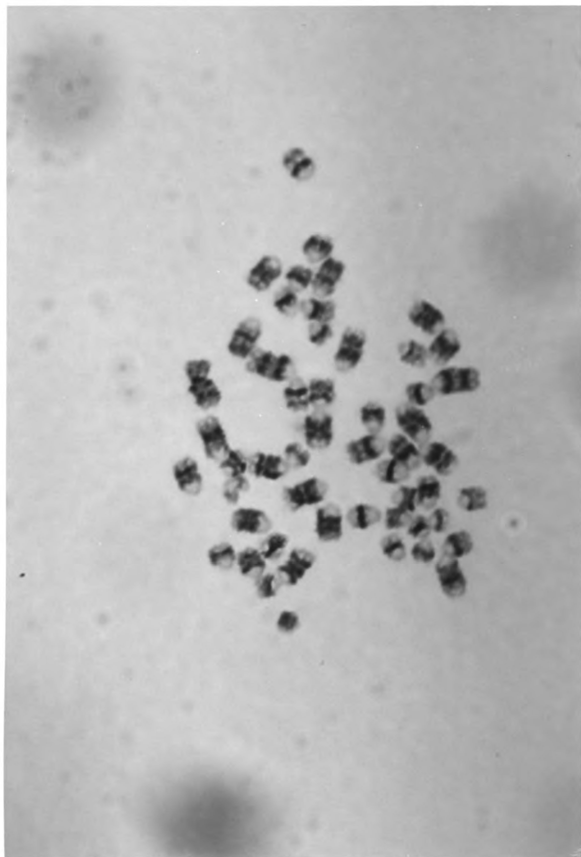
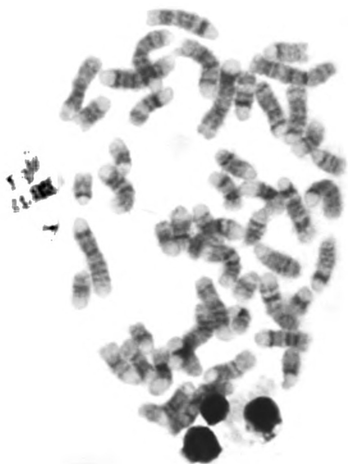


Figure 6. A <u>Bos</u> <u>taurus</u> mitoses of approximately 300 bands per haploid set of chromosomes.

Figure 7. A Bos taurus mitoses of approximately 500 bands per haploid set of chromosomes.



SUMMARY AND CONCLUSIONS

Until recently, high-resolution chromosome banding techniques were used exclusively in humans. A high-resolution chromosome preparation is much more informative than a conventional chromosome preparation. Due to the highly elongated state of the high-resolution chromosome, considerable more precision may be afforded in diagnosis of chromosomal aberrations and recognition of a normal or typical chromosome map from which to refer to.

Obtainment of highly elongated chromosomes in the human was accomplished through the use of a folate antagonist (amethopterin) which inhibits a cell at S phase of the cell cycle. The cell is released from the block with thymidine and collected at a critical time. The intricacies of the system have been thoroughly worked out in the human. Studies conducted with bovine chromosomes have not reached this level of sophistication. In this thesis a human chromosome preparation procedure was modified to obtain elongated chromosomes from *Bos taurus*.

From this study it was determined that the optimum time to collect cells after release from the amethopterin imposed block was 4.75 h. The haploid chromosome band number for *Bos taurus* at early metaphase was established as 351 by Di

Berardino (1985). Treatment determined as optimum in this study resulted in approximately 40% of all observed chromosome spreads exceeding 310 bands per haploid set.

This thesis is the only report known describing a technique to obtain high-resolution G-bands via Wright's stain in Bos taurus. This work represents a further step toward definitive characterization of the G-banding pattern of individual chromosomes of this species, refined studies of chromosomal ultrastructure, more accurate analysis of banding homologies among species and/or subspecies of the family Bovidae, comparitive evolution, molecular organization of chromosomes, detection of minute chromosomal changes in birth defects and neoplasia, and finally, the development of an accurate and highly refined chromosomal map providing useful information for the correlation of such cytogenetic maps to genetic linkage maps.

APPENDIX

Appendix 1. Stain Preparation

Stain, 1 liter preparation. Carefully weigh out 2.5 grams (+/- 0.01) of Wright's stain (Sigma) and place in a 1 liter volumetric flask. Fill to the 1 liter mark with absolute methanol. Place a stir bar in the flask. Seal the top, cover completely with foil, then stir the solution for 3 h at medium-fast speed. Using two stacked #1 filters, filter the stain and fill storage bottles up to and in to the neck of the bottles (to minimize air space). Cover bottles completely with foil. Place bottles in a 37° C incubator for 3 to 5 d to cure (stain may be used after 1 d but results may be inconsistent). Remove bottles from incubator and store at room temperature in an area sheltered from light. Depending on the stain lot, conditions during preparation of of the stain and technical precision, the unopened stain may keep for 3-12 months without significant deterioration. Once opened the stain will deteriorate relatively fast. The rate of deterioration is determined by the number of times the bottle is reopened, the amount of air-space in the bottle, and the length of time the bottle is open and exposed to the atmosphere. For these reasons, it is advisable to store stain in individual quantities of a size that will be used quickly.

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