REPLICATION MAPPING THE HUMAN GENOME

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ABSTRACT

The replication kinetics of human chromosomes employing 5-bromodeoxyuridine (BrdU) has been reported by only a few individuals. These investigations involved the use of different tissues, amniotic fluid or blood cells, and modified techniques. Consequently, the origins of the conflicting replication patterns published were indeterminate. Technical difficulties characteristic of this BrdU-replication procedure were reported to be very low mitotic indices and elongated chromosomes unsuitable for banding. This project was initiated to resolve some of the contradictory replication kinetics and the technical problems reported.

Diploid human female fibroblast line , 46,XX, was selected from which the replication map of these chromosomes could be constructed. In addition, human female fibroblast line, 46,X,del(X)(q13), was chosen which had lost a portion of the long arm of one X chromosome. This was chosen to determine whether a deletion of a chromosomal segment will alter intrachromosomal replication kinetics or the interchromosomal replication map.

Replication mapping utilizes BrdU, a base analogue of thymidine, which is incorporated into those chromosomal regions which are in the process of replicating their DNA during the synthesis (S) phase of the cell cycle. Such regions substituted with BrdU decrease the staining intensity of Hoechst or Giemsa. Consequently, regions substituted with thymidine stain darker than those substituted with BrdU. This enables the precise localization of early, middle and late S DNA replicating regions of chromosomes depending upon the time of addition

of BrdU or thymidine.

In order to obtain replication map, the toxicity of BrdU and FrdU upon fibroblasts was overcome and a new experimental protocol devised. Also, more efficient staining procedure for the photographing of sequentially Q- and Giemsa Replication (GR) banded chromosomes was developed. Utilizing these procedures a replication map of human fibroblast chromosomes from both cell lines was obtained.

Each chromosome was found to stain homogeneously dark when thymidine was incorporated for the entire S phase of that particular cell. As the duration in thymidine progressively decreased, the staining intensity of chromosomes concurrently decreased and gaps in the staining began to appear. These gaps coincided with R-bands. Presumably these regions represent the earliest areas to complete DNA synthesis. As these areas widen and increase in frequency, this results in first the appearance of Q- or G- bands and finally in a C- banding pattern.

Homologous X chromosomes were easily differentiated either by a comparison of the bands present or their staining intensity. Replication kinetics of the structurally abnormal heterocyclic X chromosome was determined to be very similar to that of the normal heterocyclic X chromosome. The X chromosome with a deletion of a portion of the long arm was consistently late replicating. These findings are consistent either with the X activation hypothesis of Comings (1968) or Brown and Chandra (1973).

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INTRODUCTION

Replication mapping of chromosomes has been developed very recently and employs 5-bromodeoxyuridine (BrdU), a base analog of thymidine, which will be incorporated into cellular DNA during synthesis (S) phase of the cell cycle. When BrdU is administered during the S phase it will be incorporated into those chromosomal regions which are in the process of replicating their DNA. Such regions with BrdU substituted for thymidine show a marked decondensation. (Zakharov, 1971, 1972, 1973, 1974). In addition, BrdU possesses the property of quenching the fluorescence of specific fluorescent dyes such as the bisbenzimidazole dye 33258 Hoechst (Latt, 1973, 1974) or decreasing the staining intensity of Giemsa (Korenberg and Freedlender, 1974; Perry and Wolff, 1974).

The interval of the S phase at which BrdU is available to a cell will determine the resulting staining of the chromosome. This makes possible the precise localization of early, middle, and late S DNA replicating regions of chromosomes. This research project describes the replication pattern of human diploid fibroblast chromosomes. In addition, another objective was to determine whether a deletion of a chromosomal segment would alter the intrachromosomal replication pattern or the interchromosomal replication map. For this purpose a human fibroblast strain SK5239 was chosen which had lost a portion of the long arm of one X chromosome. This will enable the chromosomal regions of late S DNA replication to be precisely compared.

The very nature of the substances involved, their specific incorporation into DNA of chromosomes in conjunction with the specific effect

upon dyes will enable a more precise determination of replication patterns than was previously obtainable with autoradiography.

Only a limited number of individuals have been able to report the replication pattern of human chromosomes employing a modified BrdU technique. Epplen et al. (1975), Grzeschik et al. (1975) and Latt (1975) reported only the late replicating areas while Kim et al. (1975) reported only the early replicating regions. All of these researches involved the use of amniotic fluid or blood cells. However, discrepancies exist in the replication patterns reported. In addition, inherent technical difficulties result in very low mitotic indices and poor quality mitoses (Craig-Holmes, 1975). Therefore, this project was initiated to resolve the contradictory replication patterns reported and also to overcome the technical problems reported. These are critical if the widespread applications and potential of this technique are to be realized.

LITERATURE REVIEW

In order to comprehensively review this topic, several diverse but related subjects must be examined. The cell cycle of diploid human fibroblast derived from fetal tissue must be closely approximated to obtain informative results. The metabolism of nucleotides, their incorporation into DNA and secondary cytological effects must be studied. The molecular basis of the observed staining effects of BrdU must be understood in order for an appropriate technical protocol to be determined.

The life cycle of metabolizing cells has been divided into an interphase consisting of G_1, S, and G_2 periods and a mitotic phase (Howard and Pelc, 1953). Chromosomal DNA is doubled during a defined period of interphase, the synthesis or S-phase. After mitosis and before the S period, the DNA content of the interphase nucleus is constant. This phase is called the first gap period, or G_1 period. In a similar manner, the DNA content remains constant after the termination of DNA synthesis in the S phase until the chromosomes divide in mitosis. This is the second gap period of the cell cycle or G_2 period. For a diagrammatic representation of the cell cycle refer to Fig. 1 . Therefore the S-period is preceded and followed by intervals in which DNA synthesis is normally absent although RNA and protein synthesis occur in most cell types during these intervals (Miller, 1970).

In logarithmically dividing diploid human fibroblast cultures, the duration of the S period has been reported between 7.5 to 11.5 hours and the duration of the G_2 has been reported from 4.0 to 5.9 hours,

Figure 1. Model cell cycle employed upon which the experimental protocols were based. Arrows indicate addition of chemicals in relation to the cell cycle phase for the 7 hour terminal thymidine treatment.

Figure I

DIPLOID FIBROBLAST CELL CYCLE

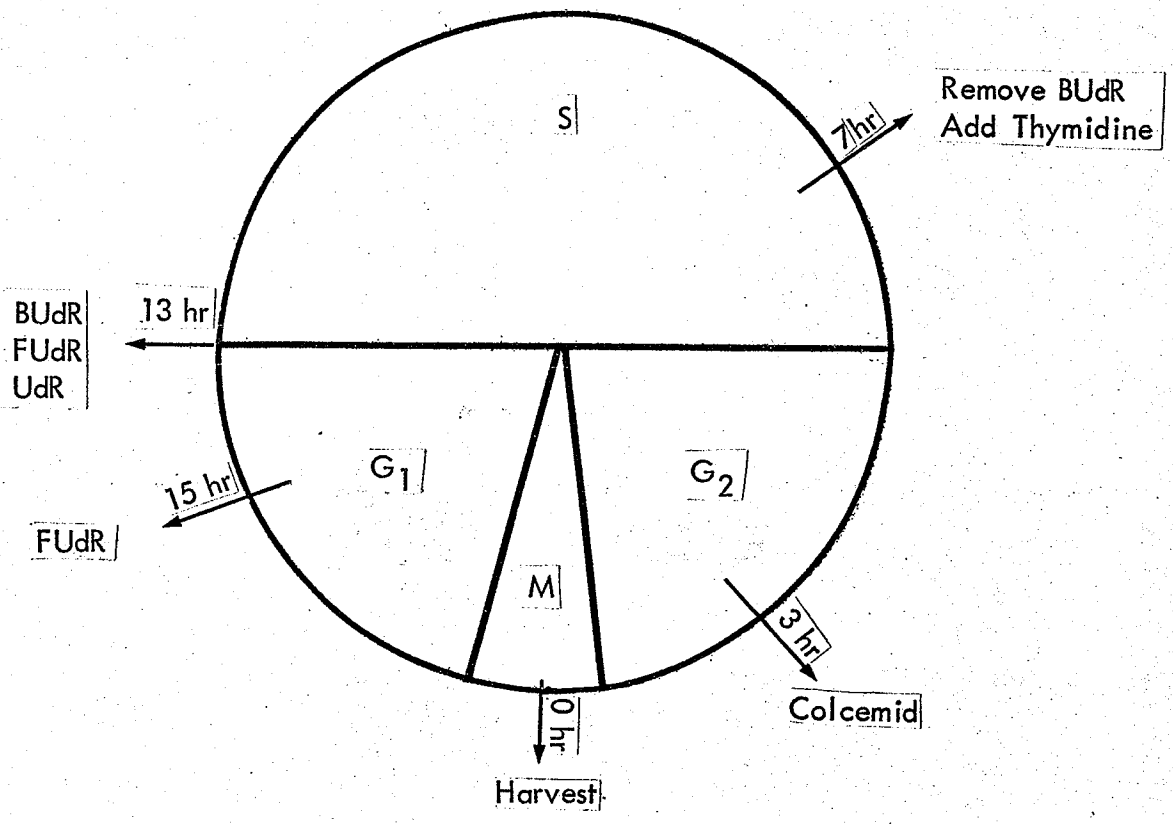


Table 1, (Defendi and Manson, 1963; Kukharenko et al., 1974; Moorhead and Defendi, 1963; Porter and Paul, 1974). Both the S and G_2 periods are less variable in duration than the G_1 in many different mammalian tissues and species (Aoki and Moore, 1970; Cleaver, 1967; Defendi and Manson, 1963; Gianelli, 1970). In a heteroploid human amnion cell line the G_1 period was the most variable portion of the cell cycle while S, G_2, and M were very constant (Sisken and Marasca, 1965). The limited variability of the duration of the S, G_2, and M phases is an essential prerequisite for the accurate determination of a replication map. Under standard experimental conditions these cell cycle parameters in diploid embryonic strains are very stable irrespective of individual differences, sex, and the number of passages throughout their logarithmic stage of growth (Kukharenko et al.,1970).

During the S period not all chromosomes and chromosome regions replicate DNA synchronously. Employing autoradiographic techniques, the heterochromatic regions were found to initiate and terminate DNA synthesis later than the euchromatic regions (Grumbach et al., 1963; Lima de Faria, 1959; Taylor, 1960). There also exist minute differences in the timing of DNA replication in different chromosome or different chromosome segments. Although autoradiographic techniques were not capable of detecting these differences, several authors have demonstrated that the rate of DNA synthesis was not constant throughout the S period (Stubblefield et al., 1967; Takagi and Sandberg, 1968). An intermittent cessation of DNA synthesis of 1 hour duration in the

Table 1. Comparison of the *in vitro* duration of cell cycle phases from a variety of fibroblast or fibroblast-like cell lines. These were determined by the use of labelled precursors and autoradiography.

TABLE 1

IN VITRO DURATION OF CELL CYCLE PHASES

HUMAN CELL TYPE	DURATION IN HOURS G_1	S	G_2	M*	T	REFERENCE
Diploid embryonic fibroblasts	2.5	11.5	4.5	-	18.5	Moorhead & Defendi, 1963
Diploid infantile fibroblasts	4.0±.96	10.6±52	5.9±.77	-	20.5±98	Porter & Paul, 1974
Diploid embryonic fibroblasts	2.4-4.6	10.15±55	5.4±.3	-	18-20	Kukharenko et al., 1974
Diploid fibroblast-like	4.5-8	7.5	4.0	-	18	Defendi & Manson, 1963
Amnion	9.8	6.8	2.2	0.6	19.4	Siskan & Morasca, 1965

* The duration of mitosis is either shown separately or included in the estimate of G_1 and G_2 phases.

middle of an approximately 9 hour S period of human epithelial adenocarcinoma cells was detected by autoradiography (Kasten and Strasser, 1966).

One of the most firmly established principles arrived at by autoradiographic labelling studies of DNA replication was that a consistent sequence or pattern of synthesis exists (Miller, 1970). Meuller and Kajiwara (1966) demonstrated this when HeLa cells were labelled with tritiated thymidine during early S, allowed several generations of growth, and then labelled with bromodeoxyuridine (BrdU) at various intervals of the S phase. Both labels were observed in the same DNA fragments. This indicated that DNA molecules replicated during the initial part of the S period are replicating in the same temporal sequence in relation to the other DNA molecules in subsequent S periods of their daughter cells. Consequently, an orderly sequence is observed at the level of whole chromosomes. Particularly towards the end of the S period a pattern exists of chromosomal regions which are still synthesizing DNA. This is called the late DNA replication pattern. Bader et al.,(1963); Bianchi and Bianchi (1965); German (1962, 1964 a and b); Morishima et al.,(1962); Schmid (1963), provided the first descriptions of this late replication pattern in human chromosomes employing tritiated thymidine and autoradiography. This was the only method of identifying individual human chromosomes beyond purely morphological methods before the advent of various banding techniques, such as Quinacrine and Giemsa banding, and therefore served as an important means of chromosome identification. Similarly, there exist characteristic

early and middle S DNA replicating chromosome regions. Asynchrony in the initiation of DNA synthesis in different chromosomes in pulse labelled cultured human leukocytes was reported by Bianchi and Bianchi (1965), and Kikuchi and Sandberg (1964). However these segments are technically harder to detect utilizing autoradiography and the pattern of initiation of DNA synthesis is not as marked as that found at the end of the S period. As a general rule, the initial labelling patterns detected by autoradiography appear to be the reverse of the terminal labelling patterns (Ockey et al., 1966; Slezinger and Prokofieva-Belgovskaya, 1966; Slezinger and Prokofieva-Belgovskaya, 1968; Sofuni and Sandberg, 1967; Takagi and Sandberg, 1968, a & b). Two analyses (Gavosto et al., 1968; Gilbert et al., 1965) show that there is no sharp time of transition between early and late replicating regions. Rather, the late replicating regions start DNA synthesis later and show higher rates of DNA synthesis than the other regions. The higher rates of synthesis are per unit length of chromosome and do not imply that the molecular rate is any higher in any one particular region. The heterochromatic regions of chromosomes, which remain condensed and more easily stainable during interphase, are always late replicating (Lima de Faria, 1969).

Recently, a new methodological approach enabling the visualization of DNA replicating chromosome regions has been developed utilizing differences in the microfluorometric properties of BrdU substituted DNA. When BrdU is administered at the proper concentration during S phase it will be substituted for thymidine in those regions which are

in the process of replicating DNA. This substitution of BrdU into the chromosomal DNA of mammalian cells has been reported by several authors (Djordjevic and Szybalski, 1960; Eidinoff et al., 1959; Hakala, 1958, 1959; Lough and Bischoff, 1976; Simon, 1963; Szybalski and Djordjevic, 1959, 1960).

Base analogues can be incorporated into DNA only if they can undergo hydrogen bonding, within the confines of the DNA double helix, to the partner of the base which they are replacing (Davidson, 1972). BrdU is a base analogue of thymidine because the van der Waal radius of bromine of 1.95 Å very closely resembles the radius of the methyl group on thymidine of 2.0 Å (Szybalski, 1962). Therefore, it is generally accepted that BrdU is converted by a salvage pathway to bromodeoxyuridine triphosphate (BrdU TP) (Kornberg, 1974). This triphosphate can compete with the naturally occurring deoxythymidine triphosphate (dTTP) and will be incorporated into DNA by DNA polymerase which accepts both BrdU TP and dTTP (Davidson, 1972; Kornberg, 1974; Lehninger, 1972).

The incorporation of BrdU into chromosomal DNA during the S phase of the cell cycle has several secondary effects upon the metabolism of cultured cells. Several hours are required before these consequences can be observed. This is unlike fluorodeoxyuridine, another base analog, whose inhibition of deoxyribonucleotide and DNA synthesis occurs immediately after exposure of the cells to FrdU (Kihlman, 1966). The concentration of Brdu, the duration of exposure, and the cell type studied all determine the effects which are observed. The growth of

mammalian cells in culture exposed to BrdU at concentrations resulting in significant substitution of BrdU in place of thymidine generally leads to cell death (Kihlman, 1966). Mammalian cells treated with bromodeoxyuridine generally have an increased frequency of chromosome aberrations. This increase depends upon the concentration of BrdU employed with some cell types increasingly sensitive to BrdU than others (Kihlman, 1966). However, mouse fibroblasts resistant to the effects of BrdU have been reported (Hsu and Somers, 1962; Kit et al., 1963). In the majority of cases, resistance results from the loss of an enzyme which is essential in the conversion of BrdU into a DNA precursor.

Another cytological effect when BrdU is incorporated into the chromosomal DNA during the S phase is the marked decondensation of chromosomes which thus appear elongated (Zakharov et al., 1970, 1971, 1972, 1974). This was observed when BrdU was administered to cultured peripheral human lymphocytes at a dose of 200 micrograms per ml. 5 - 7 hours prior to harvesting which is during the final stages of the S period of the cell cycle (Zakharov et al., 1974). When BrdU is administered to cultures of peripheral human lymphocytes at a lower concentration of 20 micrograms per ml. (Kim, 1974) or to human lung fibroblasts at a concentration of 5×10^{-6} M (Miller et al., 1976) for an equivalent or extended period of time no decondensation is observed in the chromosomes.

At these reduced concentrations BrdU is incorporated into the

chromosomal DNA without affecting the gross morphology of the chromosome. However, the chromosomal regions incorporating BrdU will display a reduced level of fluorescence when stained with specific fluorescent dyes when compared to the unsubstituted chromosomal segments (Latt, 1973). The most notable decrease in fluorescence is obtained when 33258 Hoechst is used while Quinacrine, Acridine Orange, and Proflavin show a smaller reduction in the fluorescence of BrdU substituted polynucleotide chains (Latt, 1974). In contrast, the fluorescence of daunomycin and ethidium bromide is not reduced by the incorporation of BrdU into the polynucleotide chains. This effect of BrdU upon 33258 Hoechst fluorescence in cytological preparations appears to be the result of a reduction in dye fluorescence quantum yield (Latt and Wohlleb, 1975). The quenching effect of Brdu incorporation upon Hoechst fluorescence will be abolished when the slides are mounted after staining with 33258 Hoechst at a pH below 4.0 or in a solution containing glycerol, or both. This BrdU quenching of 33258 Hoechst fluorescence provides a microfluorometric method for the differentiation of BrdU substituted DNA. As a consequence, this characteristic may be utilized to determine the replication map of the human genome when BrdU is administered to the cells during a specific portion of the S period of the cell cycle.

A significant technical improvement was achieved when BrdU treated cells were stained with 33258 Hoechst and subsequently by Giemsa banding method (Korenberg and Freedlender, 1974; Perry and Wolff, 1974; Wolff and Perry, 1974). This enabled the cytological preparation of permanently Giemsa differentiated chromosomes in contrast to the

Hoechst dye which demonstrated a remarkable degree of photo-instability so that the fluorescence faded very rapidly. Perry and Wolff's (1974) post-fluorescent staining treatment included the incubation of slides for two hours at 60 °C mounted in either 2X SSC (0.3M sodium chloride and 0.03M trisodium citrate) or water. This was followed by 30 minutes staining in 3% Gurr's R66, ph 6.8, Giemsa solution. The post-fluorescent treatment of Korenberg and Freedlender (1974) involved the incubation of slides in 1.0 M NaH_2PO_4 (pH 8.0) at 87-89 °C for 10 minutes followed by staining in 5% Harleco Giemsa for 2-10 minutes. This was similar to the previously reported techniques of Kato and Moriwaki (1972) and Sehested (1974) for different application employing non-BrdU substituted chromosomes. Both protocols yielded similar results. Bifilarly substituted DNA chromosomal segments, which have BrdU present in both strands of DNA helix, fluoresced dimly with 33258 Hoechst and stained weakly with Giemsa whereas, unifilarly substituted DNA chromosomal segments fluoresced brightly and stained darkly with Giemsa. The fluorescent plus Giemsa (FPG) technique offers additional resolution over the conventional fluorescent technique. When small double exchanges occur near the tips of chromosomes, these will be seen clearly with Giemsa whereas after fluorescent staining alone, both tips often appear to fluoresce because of flare (Perry and Wolff, 1974). Other advantages over the fluorescent technique are the permanence of the preparation, no special optical equipment is required, and the staining can be adjusted easily to treat new or old slides.

Several chemical bases of the FPG technique have been postulated. Latt (1973) found that the fluorescence efficiency of 33258 Hoechst bound to poly(dA-BrdU) was less than 1/5 of that of the dye bound to poly (dA-dT). He considered that the fluorescence of the chromatids containing BrdU substituted DNA was quenched. Zakharov and Egolina (1972) supposed that BrdU caused physicochemical alteration of DNA molecules probably impairing the synthesis of chromosome condensing proteins and therefore, causing different spiralization and Giemsa staining of the BrdU incorporated chromatid segments. Studies have shown that differentially BrdU substituted chromatids have varying densities which is apparent without heat treatment. Also, differential staining appears to reflect an underlying structural difference between the chromatids (Korenberg and Freedlender, 1974).

Ikusima and Wolff (1974) have postulated that differences in the binding of proteins to BrdU containing chromatids is the source of the underlying structural difference and therefore, the cause of the differential staining with the FPG. DNA substituted with BrdU has an increased affinity for chromosomal proteins (Gordon et al., 1973). Goto et al., (1975) and Sugiyama et al., (1976) have found strong evidence indicating that chromosomal proteins are not primarily responsible for the differential staining which they determined to be a function of the concentration of 33258 Hoechst and the duration of exposure to light. They observed that BrdU containing chromatids take up less azure dyes after Giemsa staining and this loss of accessibility

of the azure dyes to the chromatids is induced by exposure to intense light. Light activated chemical reactions alter the chromatid components preventing further binding of azure dyes to DNA either by the masking or degradation of DNA. Because urea and trypsin which markedly alter the accessibility of the dyes to nuclear DNA (Shiraishi and Yosida, 1972; Sugiyama, 1968) failed to reverse the differential Giemsa staining, proteins may not play an important role in chromatid differentiation. The results of Goto et al.,(1975)and Sugiyama et al.,(1976) indicate that the photolysis of DNA is the predominant cause of the differential staining which is consistent with the hypothesis of Kornberg and Freedlender (1974). The photolysis of differentially substituted DNA may provide the underlying structural difference which results in differentially stained chromatids.

Autoradiographic replication patterns were the only means available which could identify individual human chromosomes beyond purely morphological methods such as overall length, and position of centromere prior to the development of chromosomal banding techniques. Extensive autoradiographic descriptions of replication patterns in normal and abnormal human mitotic chromosomes exist (Bianchi and Bianchi, 1965; German, 1964 a & b; Gianelli, 1970; Miller, 1970; Passarge, 1974; Schmid, 1963). Studies comparing late DNA replicating patterns with C, G, and Q banding are of particular interest (Breg et al., 1972; Calderon and Schnedl , 1973; Ganner and Evans, 1971; Schnedl, 1973). These comparative studies all utilizing autoradiography have revealed that, in general, the late replicating chromosomal

segments correspond to the patterns of G-and Q-bands. However, it has been shown with autoradiography that the C-bands are, in general, among the latest replicating areas. C-bands correspond to heterochromatic regions which have long been known to synthesize DNA relatively late during the S period (Lima de Faria, 1959). Autoradiographic studies using ^{3}H-thymidine (Pera, 1968) and studies of premature chromosome condensation (Sperling and Rao, 1974) have both demonstrated that the heterochromatic regions initiate and terminate DNA replication later than the euchromatic regions.

Quinacrine dyes stain AT rich DNA chromosomal segments (Dev et al., 1972; Ellison and Barr, 1972; Schreck et al., 1972; Weisblum, 1973) and tritiated thymidine will be incorporated preferentially into these same regions. This could result in the labelling of AT rich Quinacrine positive bands even if no asynchrony of DNA replication existed. In an attempt to determine if the correlation between ^{3}H-thymidine incorporation and Q positive bands is merely a reflection of the distribution of AT rich DNA within the chromosome, two authors employed ^{3}H-deoxcytidine as a radio active label (Schnedl, 1973; Zakharov, 1974). This base analogue would be expected to preferentially label GC rich areas of chromosomes and thus avoid any accidental correlation between Quinacrine positive bands and late replicating chromosomal segments. The same, but less distinct, late labelling patterns were obtained. The preferential incorporation of ^{3}H-thymidine into AT rich bands may enhance the late labelling pattern.

However, G and Q positive areas complete their DNA synthesis later than negatively stained regions. The Quinacrine positive areas, including the distal portion of the Y as well as the only dull fluorescing regions of human chromosomes, can be labelled both with ^{3}H - thymidine and with ^{3}H - deoxycytidine. Therefore, there are no detectable regions in the human chromosomes which are composed solely of AT or GC containing DNA (Schnedl, 1973) and there are no late replicating chromosomal regions predominantly composed of GC or AT base pairs to an extent which would be detected by methods currently employed (Zakharov, 1974). Zakharov et al.(1974) also compared the differential spiralization which was induced by administering bromodexycytidine and bromodeoxyuridine in the final stages of the S period of the cell cycle. The pattern of chromosomal differentiation was identical when either BrdC or BrdU was employed. Although the pattern of thymidine labelling or BrdU incorporation may be modified by the relative AT composition of various chromosomal regions, this does not significantly alter their usefulness in detecting early, middle, or late replicating chromosomal regions with the sensitivity of these techniques.

Several authors have reported the replication patterns of human chromosomes employing BrdU incubation of cultured blood cells (Egolina and Zakharov, 1976; Grzeschik et al., 1975; Kim et al., 1975; Latt, 1974, 1975; Willard and Latt, 1976) and of amniotic fluid cells (Epplen et al., 1975; Epplen and Vogel, 1975). Chromosomal segments

which replicate their DNA during the late S phase were found by these individuals to correspond closely to the pattern of G- and Q-bands. However, exceptions exist with certain G-bands such as, 3p14, 3q13, and the centromeric heterochromatin of chromosome 8, terminating replication quite early during the S phase. Additional bands, 1p36, 8q24, 12q24, 16p13, 19q13, Xp22, which stain lightly with Giemsa, are late replicating (Epplen et al., 1975; Epplen and Vogel, 1975). The patterns of late replication demonstrated only limited variation between individuals with these variable regions restricted to known regions of Quinacrine fluorescent polymorphism such as the variable bands of chromosome 3 and 13 (Latt, 1975). Chromosome 4 has the distinction of being the autosome with the most DNA synthesis during the late S phase with three very late replicating bands; p15, q13, and q28. Although the use of BrdU at concentrations employed for replication mapping may be toxic to cells (Djordjevic and Szybalski, 1960; Hsu and Somers, 1961). This is not believed to alter the relative replication map of chromosomal seqments (Latt, 1975). However, it is likely that the overall duration of the cell cycle is increased following exposure to BrdU. The late replicating chromosomal regions revealed by the BrdU-Hoechst or BrdU-FPG techniques are consistent with those detected by autoradiography (German, 1964 a & b; Gianelli, 1970; Lima de Faria et al., 1961; Miller, 1970; Passarge et al., 1969; Schmid, 1963; Takagi and Sandberg, 1968).

Kim et al. (1975) reported that early replicating chromosomal

segments coincide with R-bands. Chromosomes 4, 13, 18, 21 are among the last chromosomes to start replication along with the heterocylic X which is the last to initiate DNA replication. The speed of replication does not vary significantly between bands with the exception of the late replicating X chromosome whose rate of DNA synthesis is significantly faster (Kim et al., 1975). This is in close agreement with the results of Gilbert et al. (1965) who determined that the late replicating X synthesizes DNA at a faster rate than the autosomes using autoradiographic techniques.

The replication map of the sex chromosomes of female cells has been extensively described with autoradiography (Gavosto et al., 1967; German, 1964 a; Gianelli, 1963, 1970; Knight and Luzzatti, 1973; Petersen, 1964; Pfeiffer et al., 1965; Priest et al., 1967; Sofoni et al., 1967), 33258 Hoechst (Latt, 1974; Willard and Latt, 1976), and the fluorescent plus Giemsa technique (Grzeschik et al., 1975; Epplin et al., 1975). All three methods reveal very similar replication map of the X chromosome. With autoradiography, three patterns of the late replicating X were considered to be the most significant; X chromosome uniformly labelled, centromere unlabelled, and finally, the labelling localized to the proximal part of the long arm of the X chromosome. The correlation obtained among these labelling patterns and total grain count indicate that the former represent three successive stages of DNA synthesis with the last labelling pattern indicating the latest replicating band of the heterocyclic X. The microfluorometric determination of X chromosomal regions replicating late in the DNA synthesis

phase is generally consistent with autoradiographic results. However, use of BrdU and 33258 Hoechst or fluorescent plus Giemsa enabled an increased differentiation between the iso- and the heterocyclic X chromosome and afforded much higher resolution of replicating bands. For example, the region of late DNA replication in the proximal part of the long arm observed by autoradiography is further resolved into two components, one at Xq21 and the other at Xq23 (Latt, 1974, 1975; Willard and Latt, 1976).

Studies with the FPG technique revealed that both X chromosomes when compared to the autosomes terminate replication at the very end of the S period (Epplen et al., 1975). However the differences observed in the late replicating segments of the X chromosomes do not appear to result from simply a phase difference in the initiation and termination of replication as in the autosomes. There appears to be an actual difference in the sequence of replication which probably reflects a basic difference maintained throughout the entire cell cycle (Grzeschik et al., 1975). This may be related to the appearance of the sex chromatin during interphase.

The alteration in replication pattern, the heteropycnosis during interphase, and the resulting genetic inertness of the late replicating X have been widely associated as interrelated phenomena. Similarly, tissue differentiation has been associated with differences in the activity of gene loci which consequently may be reflected in different replication maps of chromosomes derived from different tissues.

The majority of autoradiographic studies have not revealed any obvious differences between human blood and fibroblast chromosomal replication maps (Atkins et al., 1963, 1965, 1966; Atkins and Santesson, 1966; Bianchi and Bianchi, 1965; German, 1964 a & b; Gianelli, 1970; Miller, 1970; Moorhead and Defendi, 1963; Passarge et al., 1969; Schmid, 1963; Takagi and Sandberg, 1968). However, German and Aronian (1971) noticed differences between the late replicating chromosomal segments of human lymphocytes and epithelial cells derived from mesodermal and ectodermal embryonic tissue respectively. They postulated that this was a reflection of their different cellular function in vivo, and their appearances and growth characteristics in vitro. Slezinger and Prokofieva-Belgovskaya (1968), and Slesinger et al. (1974) compared early and late replication pattern of leucocytes and embryonic fibroblasts. The chromosomes of both cell types began DNA synthesis synchronously however, chromosomes 1, 16, 21, and 22 labelled more intensely in fibroblasts while leucocyte chromosomes 4 and 5 were labelled more intensely with a terminal pulse of ^{3}H-thymidine. These intensely labelled regions would correspond to areas of late replicating DNA. This replication map is very similar to that obtained by German and Aronian (1971). Utakoji and Hsu (1965) also obtained convincing evidence that the replication pattern of the sex chromosomes differs between somatic and germ line cells of the Chinese hamster. With the poor resolution that autoradiography affords it is not surprising that the tissue specific replication patterns were not reported more frequently.

Only the investigation of Epplen et al. (1975) has studied the replication patterns of dissimilar tissues utilizing the BrdU-FPG technique. They found no recognizable differences in the replication maps of three types of amniotic fluid cells and fibroblasts. However, a comparison of the replication map of amniotic fluid cells (Epplen et al., 1975; Epplen and Vogel, 1975) and lymphocytes (Grzeschik et al. 1975) reveals several major discrepancies (Table 2). However, none of these studies can be considered conclusive due to the different techniques utilized by these researchers and none have comparatively studied the chromosomal replication maps of several tissues using the same technique. In order to resolve some of the contradictory results of the FPG technique, which may be related to the different techniques and the different tissues employed, and the inherent technical problems, such as low mitotic indices, this study was initiated.

Table 2. Late replicating bands listed for one author were absent from the late replication map of the other author. Unlisted chromosomes had no significantly different pattern of late DNA replication.

TABLE 2

DISCREPANCIES OF LATE REPLICATING BANDS OF HUMAN CHROMOSOMES

CHROMOSOME	EPPLEN ET AL., 1975* EPPLEN & VOGEL, 1975*	GRZESCHIK ET AL., 1975**
1	p36, p33, q22, q32	q31, q41
2	q31	q24, q32, q36
3	q28	p14, q13
6	p24, q14	--
7	--	q11
8	p21	--
12	q24	--
13	--	q33
14	--	q23
16	p13	p12
17	--	p12, q12, q24
19	q12, q13	--
20	q11, q13	p12

* Amniotic Fluid Cells

** Lymphocytes

MATERIALS AND METHODS

Diploid human fibroblast cell strain, SPF-46,XX was derived from a fetal skin biopsy while strain SK5239 - 46,X,del(X)(q13) was obtained from skin biopsy of patient with atypical Turner Syndrome. Both strains were stored in liquid nitrogen at -196 ^{o}C in media containing 8% glycerol until use. Culturing of these fibroblasts was according to standard laboratory procedures. Cells were utilized for experimentation exclusively during their logarithmic stage of growth.

Demonstration of early to late replicating chromosomal regions of diploid fibroblast strain SPF was achieved by the following protocol (Figure 1, Table 3). During the late G_1 and early S phase fibroblasts were cultured in fluorodeoxyuridine supplemented media for two hours at a concentration of 0.10 µg/ml. Subsequently, cells were grown in BrdU, FrdU, and Uridine supplemented media for a portion of the S phase varying from 2 to 8 hours at a concentration of 3.0 µg/ml, 0.05 µg/ml, and 2.0 µg/ml, respectively. The cultures were rinsed twice with Gibco McCoys 5A medium with 10% fetal bovine serum and this mediun supplemented with 5.0 µg/ml thymidine was added until the cells were harvested 11 to 5 hours later. Colcemid (0.05 µg/ml) was added for the last 3 hours of culture. Control received identical treatment with the exception that they did not receive a terminal thymidine pulse. Demonstration of late replicating chromosomal regions in the strain SK 5239 was achieved by a similar protocol with the number of treatment groups reduced to one, a 7 hour terminal thymidine pulse. Harvesting was according to standard technique with

Table 3. The control was handled similarly to the other treatment groups except that no terminal thymidine pulse was administered to these cells.

TABLE 3

PROTOCOL FOR GIEMSA REPLICATION MAPPING

FrdU (hr)	BrdU + FrdU + Uridine (hr)	Thymidine (hr)	Colcemid (hr)	Total (hr)
2	13	-*	3	15
2	2	11	3	15
2	4	9	3	15
2	6	7	3	15
2	8	5	3	15

* Control

the exception that the cells were left in hypotonic for 40 minutes. Cells were left in methanol: acetic acid (3:1) fixative overnight and washed at least twice in fix before spreading on slides rinsed with fixative and air drying.

Metaphase chromosomes were visualized by two staining procedures. Slides were stained initially either in 33258 Hoechst in distilled water (5µg/ml) for 15 minutes and mounted in distilled water. The alternate protocol involved staining in Quinacrine dihydrochloride (5.0 mg/ml) for 30 min. followed by differentiation and mounting in McIlvaines (pH4.5). These slides were exposed to 15 watt U.V. light at a distance of 24 inches overnight. Subsequently the slides were rinsed in distilled water and allowed to dry. Incubation in 1.0 M NaH_2PO_4 buffer (pH 8.0) for 10 minutes at 84-85°C followed and staining in 4% Gurrs (pH6.8) Harleco Giemsa solution for 4 to 6 minutes depending upon the age of the slides. Photographs were taken on Kodak High Contrast Copy film using Zeiss Photomicroscope. In order to obtain sequentially stained chromosomes these slides were destained by the following procedure: xylene 1-2 hours to remove the coverslip, xylene II - 5 minutes, 3 xylene: 1 absolute alcohol - 3 minutes, 1 xylene: 1 absolute alcohol - 3 minutes, 1 xylene: 3 absolute alcohol - 3 minutes, absolute alcohol - 3 minutes and fixative (3 methanol: 1 acetic acid) - 5 minutes. Slides were stained with Quinacrine dihydrochloride according to standard procedure (Douglas et al., 1973) with the exception that the staining time was increased to 40 minutes and differentiation time was decreased to 1, 1, and 5

minutes. The same metaphases were located and photographed with H and W film employing Zeiss fluorescent microscope with KP 500 excitation filter and 53-0-44 barrier filters. Developing and printing was performed according to standard photographic procedures.

For the identification of sex chromatin the standard protocol with Cresyl violet was followed with the exception that the staining time was increased to 30 minutes. In addition, Acridine orange was employed to identify sex chromatin. Slides were rinsed in Sorensen's buffer (pH 6.5) for 5 minutes, Acridine orange (0.05 mg/ml) for 20 minutes, Sorensen's buffer I for 1 minute, Sorensen's buffer II for 2 minutes, and mounted in Sorensen's buffer. These slides were photographed with the same procedure as previously outlined.

Chromosomes were identified on the basis of their Q-banding. In order to establish the replication pattern, a minimum of 20 homologous pairs of each chromosome were examined from each treatment group. The Giemsa replication banding patterns were recorded and then arranged sequentially so that progressively fewer replicating areas were observed.

RESULTS

The administration of BrdU, FrdU, and Uridine had multiple effects upon fibroblasts studied. Several experimental protocols were tested before the most appropriate procedure, as outlined in the materials and methods, was determined (Table 4). Initially, continuous culturing of cells for 3 or more S phases (Table 4 , Expt. 3 to 15), approximately 5 days, was attempted so that chromosomal DNA would be bifilarly substituted with BrdU except where thymidine was incorporated during the terminal thymidine exposure. This was in accordance with the fluorescent plus Giemsa (FPG) technique of Korenberg and Freedlender (1974) who achieved better sister chromatid differentiation with bifilarly and unifilarly BrdU substituted chromatids as compared to unifilarly BrdU substituted chromatids and unsubstituted chromatids. Only the former case would be attained with this initial protocol.

Because of the secondary effect of BrdU increasing the duration of the cell cycle several different periods of exposure to BrdU were tested, from 4 to 7 days. In conjunction with this, the concentration of BrdU was varied from 15 µg/ml to 1.5 µg/ml in order to overcome its inhibitory mitotic effect. However, after such prolonged exposure to BrdU even at very low concentrations a negligible mitotic index resulted which precluded the determination of the fibroblast replication map.

In the few metaphases obtained, differentially stained chromatids were consistently observed whereas banded chromosomes were unobtainable (Fig.2). Occasionally, differentially stained and banded chromatids were observed .

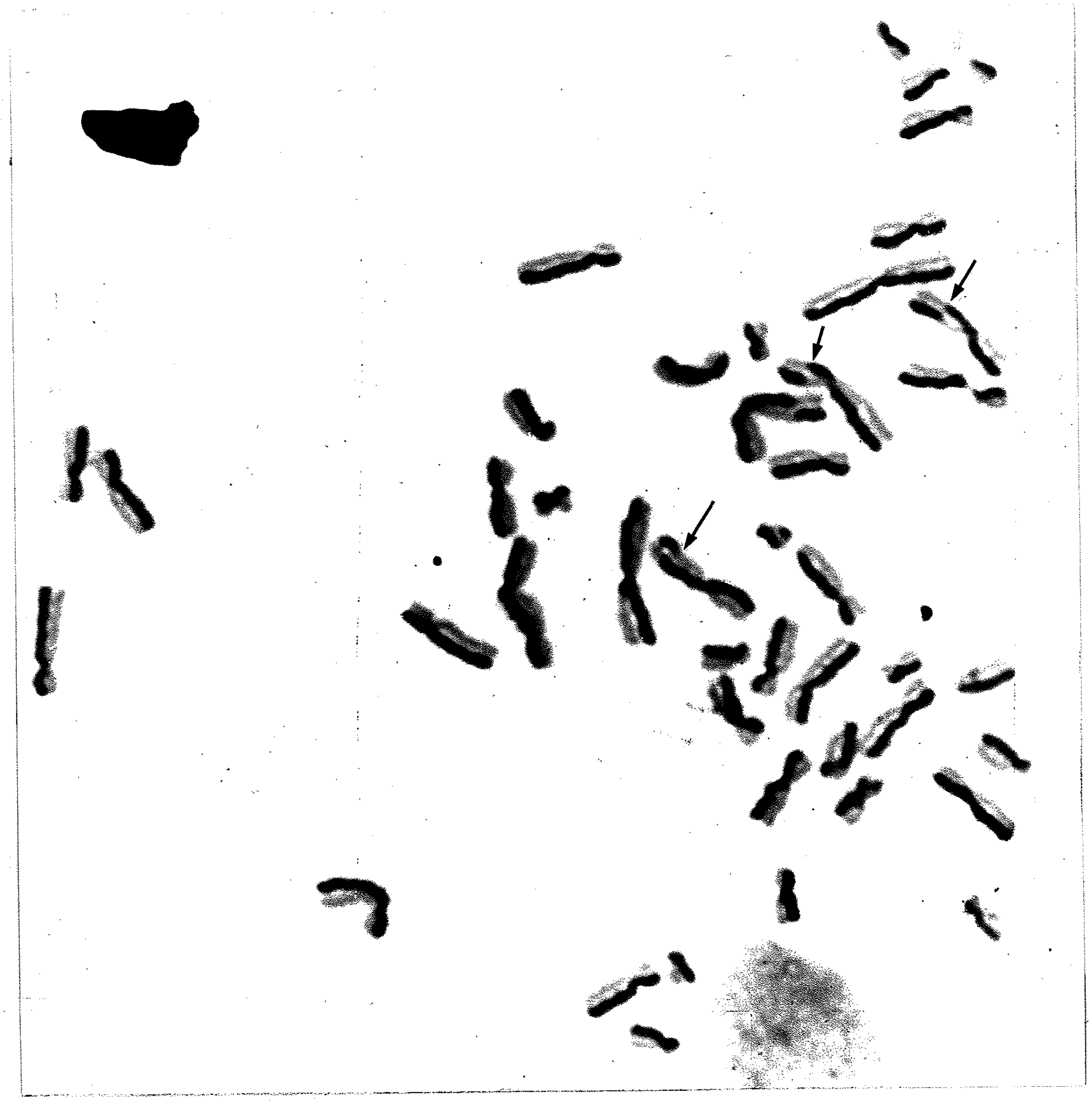
Table 4. The reduced mitotic indices obtained from these experiments reflected the toxicity of BrdU (Expt. 3 - 11) and of FrdU (Expt. 8 - 11 and 12, 13) over a period of 4 to 7 days.

TABLE 4

COMPARISON EXPERIMENTAL PROTOCOLS

Experiment Number	FrdU Pretreatment (Hours)	Exposure Time	BrdU Concentration (µg/ml)	FrdU Concentration (µg/ml)	Uridine Concentration (µg/ml)	Exposure Time (Hours)	Thymidine Concentration (µg/ml)	BrdU Chase (Hours)	Mitotic Index	Observed FPG Staining
3	--	5 days	15	--	--	5	10	4	Very low	Differentially stained chromatid
4	--	6 & 7 days	3	--	--	5	10	4	Very low	Differentially stained chromatid
6	--	4 days	3	--	--	5	10	4	Very low	Differentially stained chromatid
8-11	--	5 days	1.5	--	--	5	5	4	Very low	Differentially stained chromatid, few banded
12,13	--	5 days	1.5	0.05	1.0	5-10	5	--	Nil	--
14,15	--	4 days	1.5	0.05	1.0	5-8	5	--	Nil	--
18	1.5-3.5	2-8 hours	3.0	0.05	2.0	5-11	5	--	High	Banded chromosomes only

Figure 2. Differential Sister Chromatid Staining. Arrows indicate sites of sister chromatid exchange. These chromosomes were grown in BrdU (1.5 µg/ml) for 4 days and administered thymidine (10 µg/ml) for 5 hours followed by a 4 hour BrdU chase. No thymidine was incorporated from the terminal pulse as no bands are visible.



However, the differential staining resulting from thymidine incorporation or sister chromatid exchanges was identical and could be misinterpreted easily (Fig. 3). Therefore, such chromosomes were unsuitable for replication mapping.

In addition to reducing the mitotic index, BrdU also resulted in a decondensation of metaphase chromosomes. In some instances, this hampered the adequate spreading of chromosomes and impaired their Quinacrine and Giemsa replication banding.

However, when the fibroblasts were exposed to BrdU, FrdU, Uridine, and Thymidine for only one S phase, the mitotic index was adequate for replication mapping purposes. When the protocol outlined for both fibroblast lines was followed, banded chromosomes were observed in all treatment groups with the exception of the controls (Fig. 4). The controls received no terminal thymidine pulse and bands were not expected to be visible, only the paracentromeric areas have stained to any extent.

Numerous staining procedures were examined to determine which would prove most efficacious. Prestaining with 33258 Hoechst and Quinacrine followed by overnight exposure to U. V. light was compared with both Korenberg and Freedlender's (1974) FPG technique and Perry and Wolff's (1974) SSC Giemsa technique. After Quinacrine prestaining, photography was attempted repeatedly in order to attain sequentially Q-and GR-banded chromosomes. All of these protocols were unsuitable for the production of sequentially stained chromosomes and the

Figure 3. Differentially Stained and Banded Sister Chromatids. Arrows indicate possible sites of sister chromatid exchange. Thymidine incorporation has resulted in banding superimposed upon that of sister chromatid differentiation.

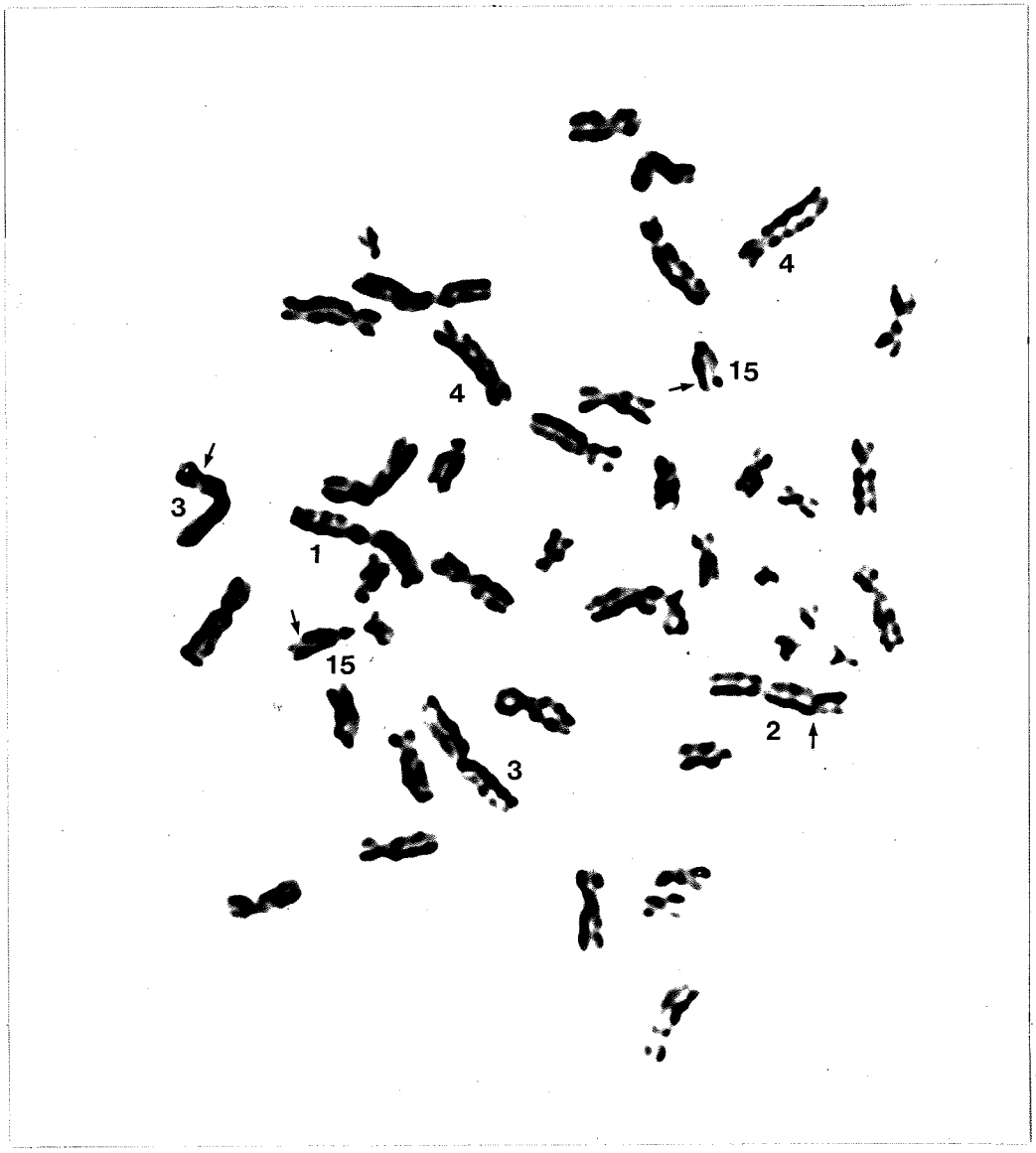
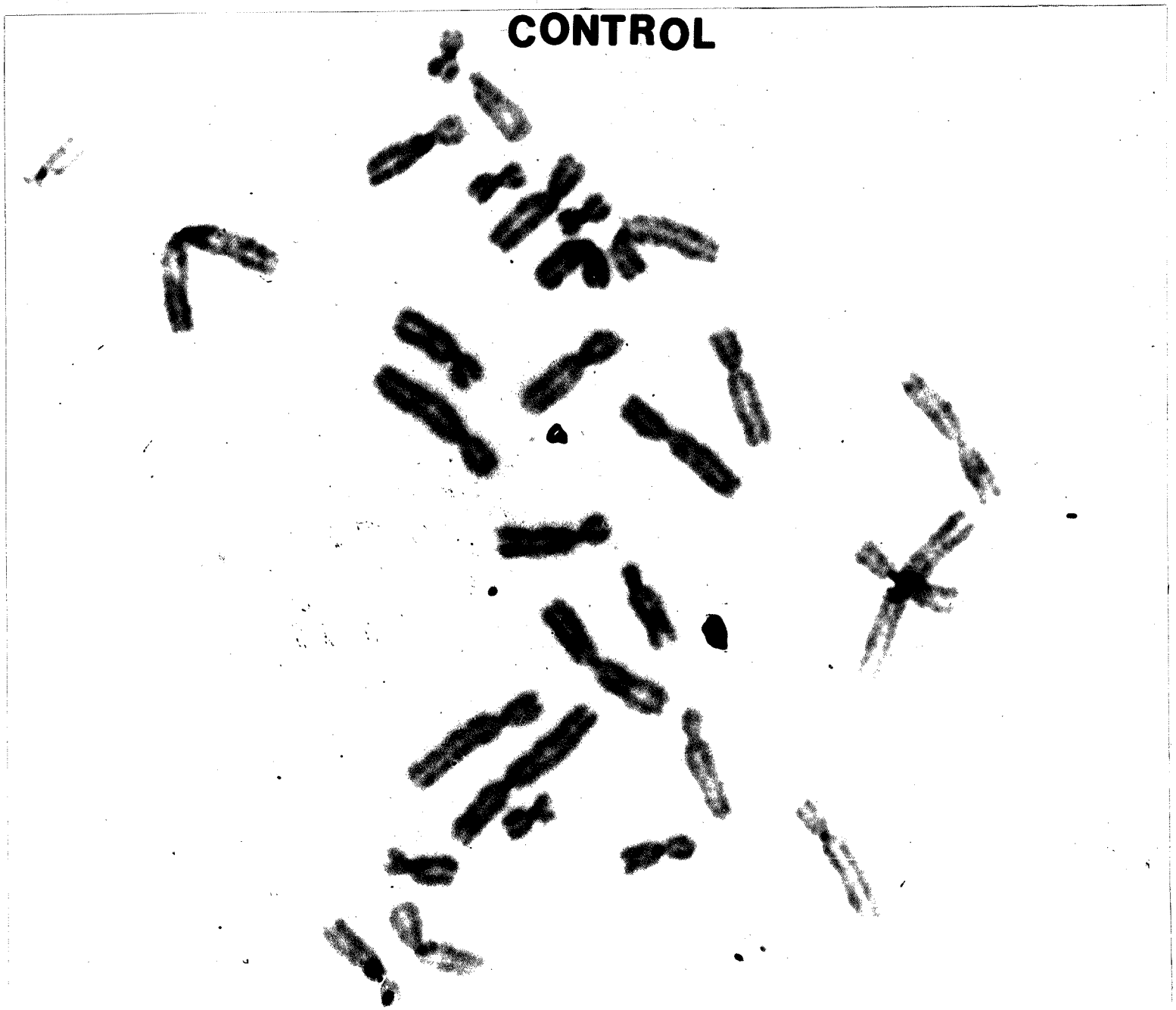

4
15
4
3
1
15
2
3

Figure 4. Replication Mapping Control Treatment Cells were not administered thymidine and no bands were observed. Only paracentromeric heterochromatin was stained.


CONTROL

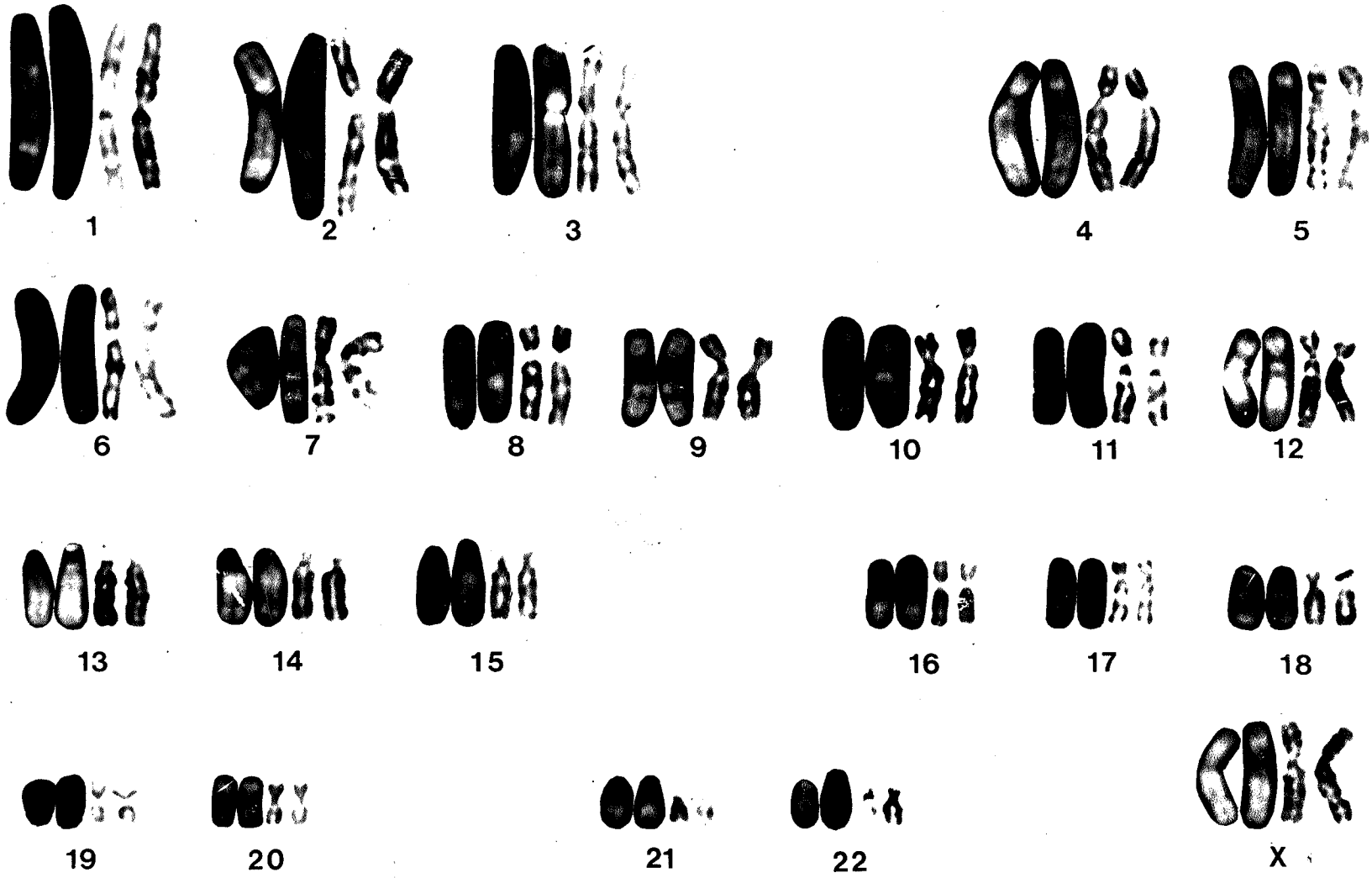
procedure described in the materials and methods section was determined to be most efficient.

The karyotypes presented for the 11, 9, 7, and 5 hour treatment groups for both the 46,XX and 46,X,del(X)(q13) fibroblast lines (Figs. 5 , 6 , 7 , 8 , 9) are composites constructed from several cells of each treatment group of each cell line. Although these chromosomes were selected on the basis of their spreading, morphology, and Giemsa replication banding, they are representative of each treatment group.

The chromosomes from the 11 hour terminal thymidine pulse (Fig. 5) demonstrates several interesting features. Several cells were obtained whose entire chromosome complement stained uniformly dark. This indicates that thymidine was incorporated along the entire length of the chromosomes. In the remaining cells, regions along the chromosomes did not stain and therefore, must have completed DNA synthesis before the addition of thymidine. Regions which are consistently early in DNA replication such as 3p27, 7q22 and 7q32 are particularly noticeable in Fig. 5 . In addition, Table 5 provides a comparison of the early replicating bands with those of Kim et al. (1975). It is characteristic of this treatment group that the homologous X chromosomes stain uniformly and the early and late replicating X chromosomes cannot be differentiated except in a few instances.

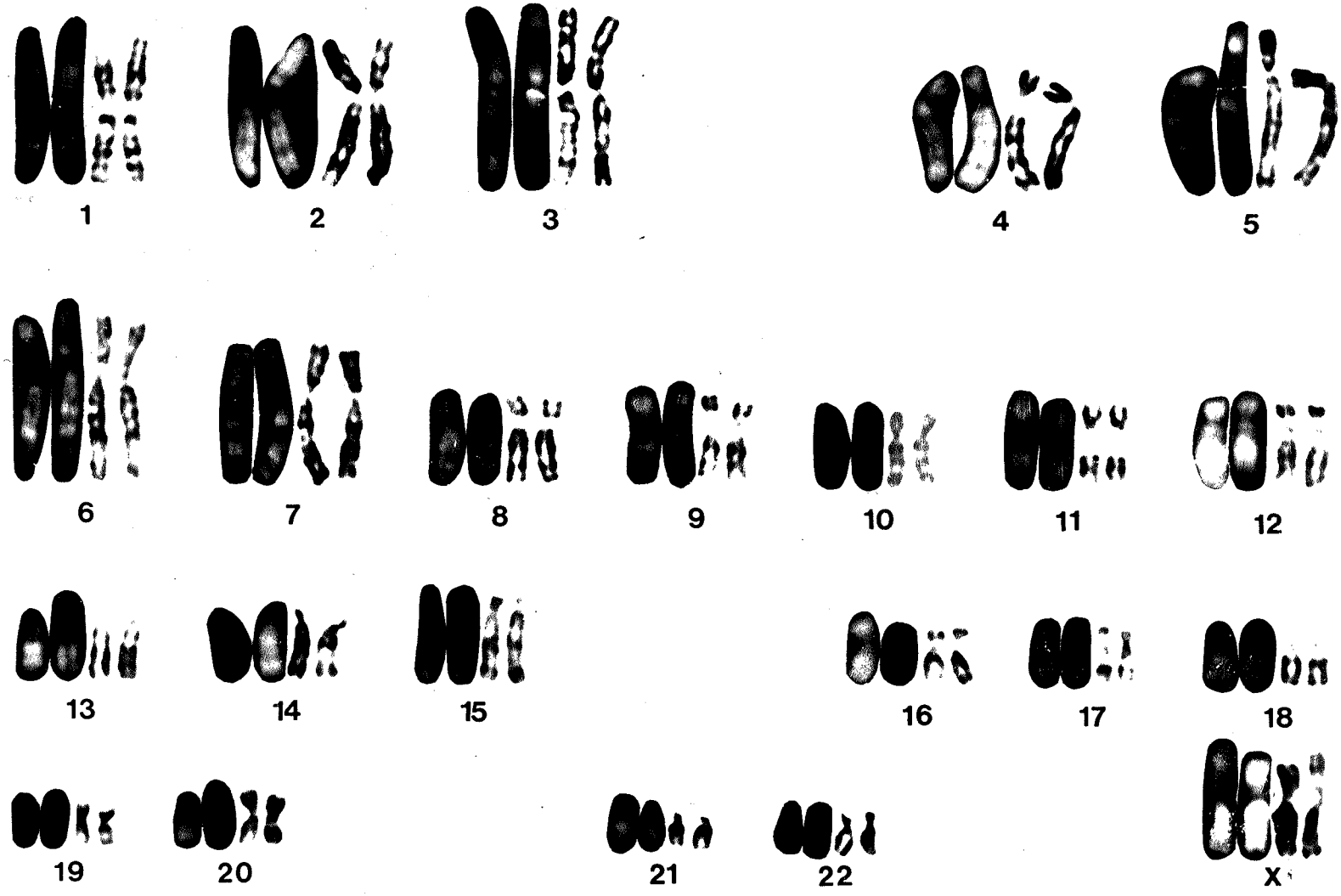
When a thymidine pulse of 9 hours is administered (Fig.6) the bands observed, in general, closely approximate those obtained with standard Quinacrine and Trypsin Giemsa staining. However, there are a

Figure 5. Karyotype Diploid Fibroblast chromosomes. Composite of homologous chromosomes constructed from the cells of the 11 hour treatment groups. Each homologous pair was identified on the basis of Q- bands after Giemsa Replication staining.



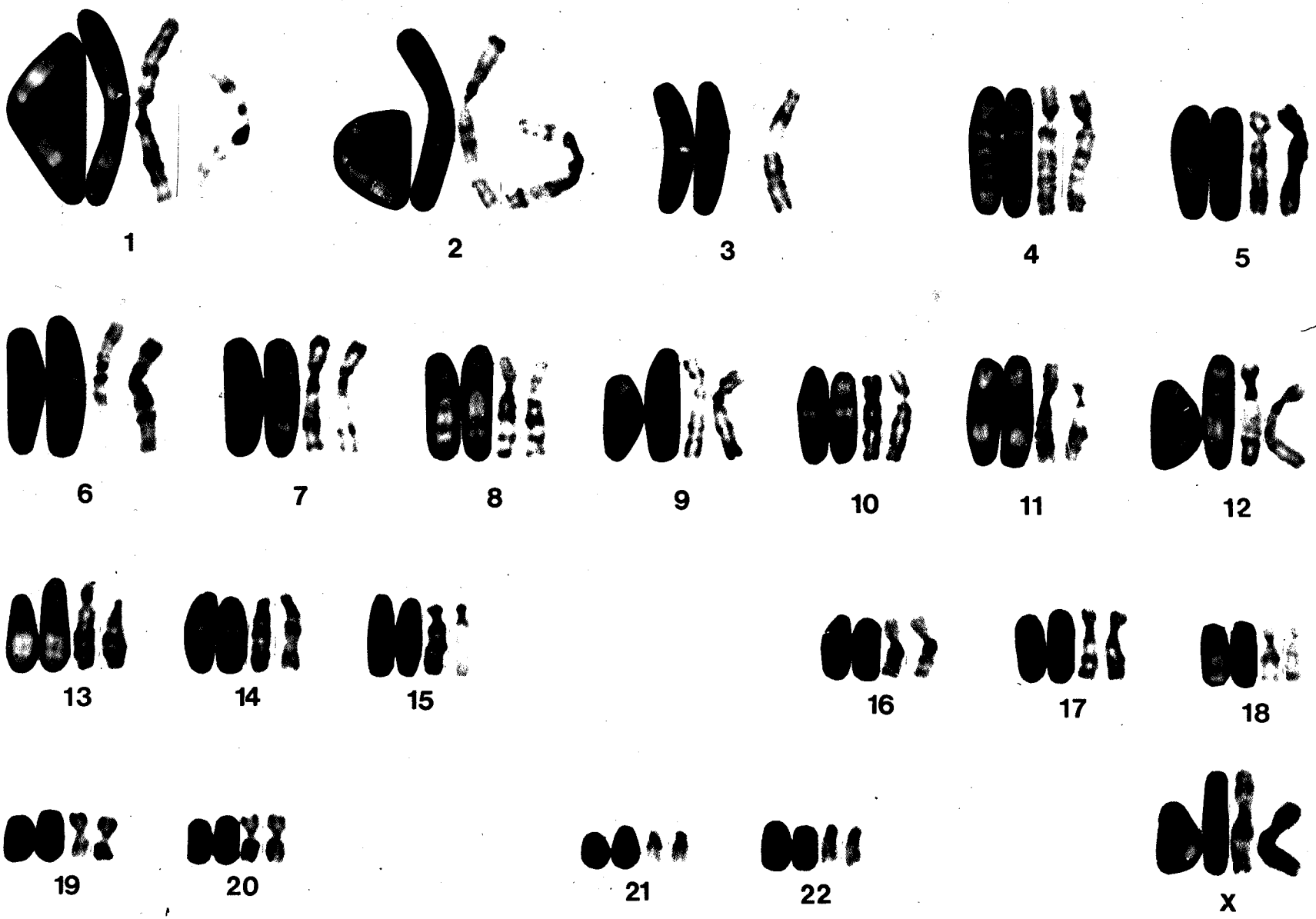
11 HOURS

Figure 6. Karyotype Diploid Fibroblast Chromosomes. Composite of homologous chromosomes constructed from cells of the 9 hour treatment group. Each homologous pair was identified on the basis of Q-bands after Giemsa Replication staining.



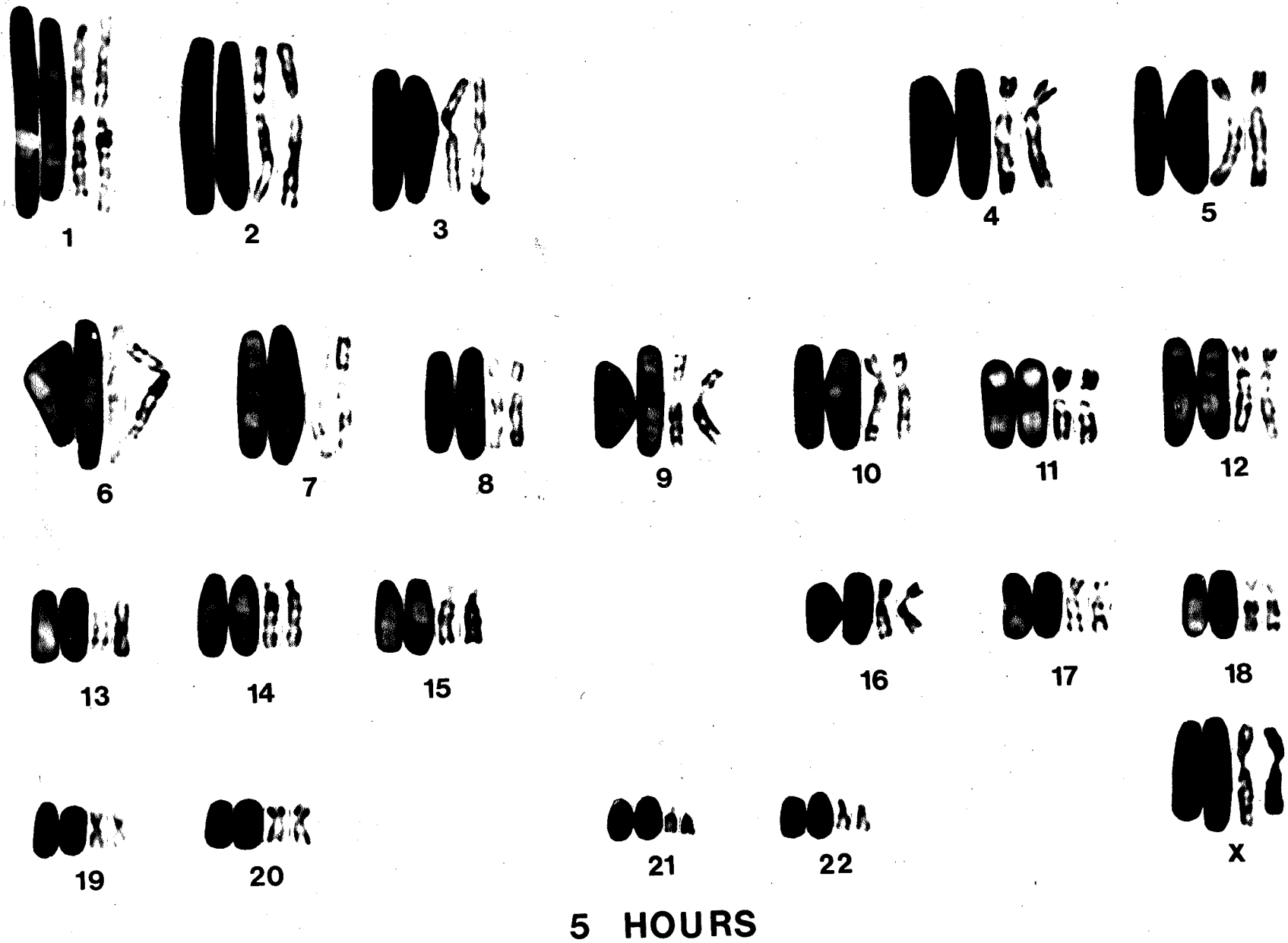
9 HOURS

Figure 7. Karyotype Diploid Fibroblast Chromosomes. Composite of homologous chromosomes constructed from cells of the 7 hour treatment group. Each homologous pair was identified on the basis of Q-bands after Giemsa Replication staining.



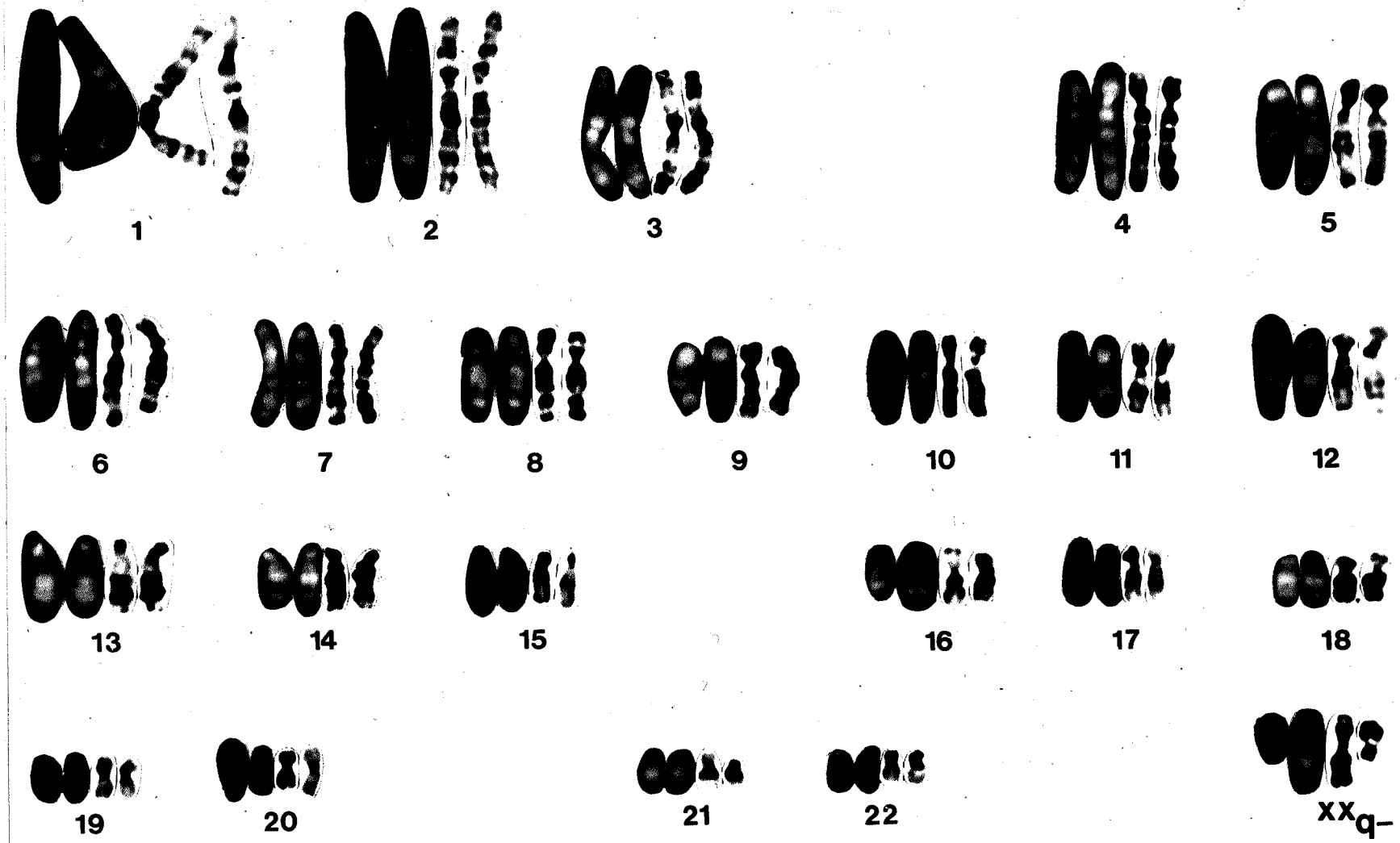
7 HOURS

Figure 8. Karyotype Diploid Fibroblast Chromosomes. Composite of homologous chromosomes constructed from cells of the 5 hour treatment group. Each homologous pair was identified on the basis of Q-bands after Giemsa Replication staining.



5 HOURS

Figure 9. Karyotype 46,X,del(X)(q13), Fibroblast Chromosomes. Composite of homologous chromosomes constructed from cells of the 7 hour treatment group. Each homologous pair was identified on the basis of Q-bands after Giemsa Replication staining.



7 HOURS

Table 5. Early replicating bands listed for one author were absent from the early replication pattern of the other author. Unlisted chromosomes had no significantly different pattern of replication.

TABLE 5

DISCREPANCIES OF EARLY REPLICATING BANDS OF HUMAN CHROMOSOMES

CHROMOSOME	KIM ET AL., (1975)*	PRESENT RESULTS**
1	pter, p34, p32	--
2	p23, p13, q13, q33, q35→qter	q31
3	p25, q27, q29	q25
4	p16	--
5	p13, q35	p11
6	q23, q25, q27	--
7	q34, q36	--
8	q11	--
9	p13, q22, q32, q34	p11, q11, q13
15	q24	--
16	p11	q13
17	q11	--
18	q11	--
20	q11	--
21	qter	q11

* Lymphocytes
** Fibroblasts

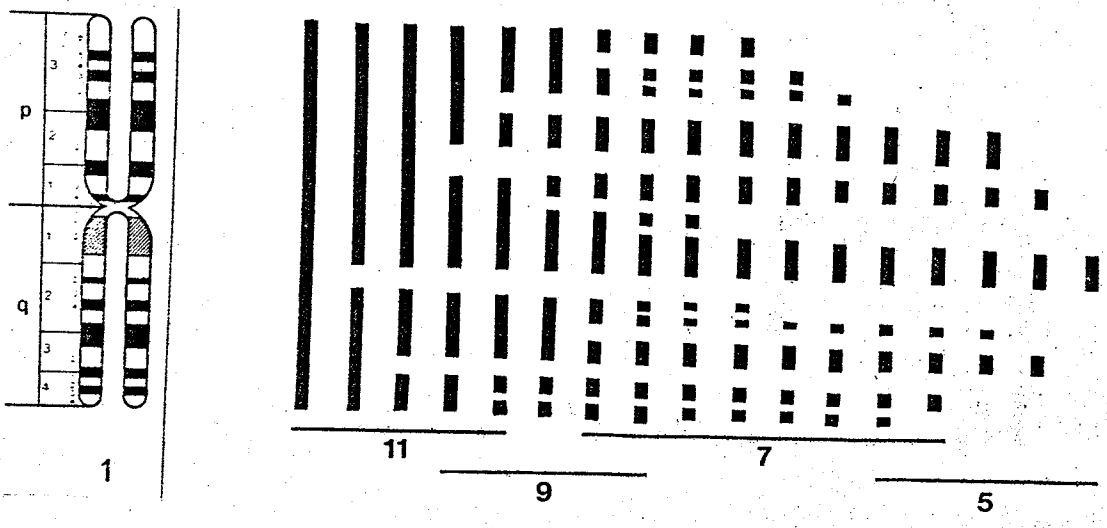
few notable exceptions. The telomeric regions of chromosomes 16p, 19q, and Xp demonstrate late DNA replication even though these regions are Q-and G-negative. The chromosomes from this treatment group have sharper and well defined bands when compared to those of the 11 hour group. Also, the non-banded regions stain less intensely than those in the 11 hour group.

The bands visualized with a 7 hour terminal thymidine pulse Figs. 7 , 9) represent late DNA synthesizing chromosomal regions. The late replication of one X chromosome and the paracentromeric heterochromatin on chromosomes 1 and 16 is consistently conspicuous. Several late replicating segments are present which do not correspond to Q- or G-bands. These are situated in the telomeric regions of chromosomes 2p, 8q, and 12q. Asynchrony in the termination, and presumably in the initiation of DNA synthesis between homologous chromosomes is frequently observed. In Fig. 7 , this is particularly striking in the case of chromosomes 1, 3 and 6. However, this asynchrony is not characteristic of, nor restricted to, these particular chromosome pairs but varies among cells.

In the 5 hour treatment group bands can be observed in some cells (Fig. 8) while only the characteristic C-bands are visible in the remaining cells. This is a consequence of the asynchrony of the cell population under examination.

On the basis of all the chromosomes studied from all treatment groups of both fibroblast lines, a replication map was constructed (Fig. 10). Several relevant points must be emphasized. Each chromo-

Figure 10. Replication Map Human Fibroblast Chromosomes. Constructed from the analysis of at least 20 homologous pairs from each treatment group for each chromosome. Horizontal lines indicate replication patterns found within each treatment group of 11, 9, 7, or 5 hour terminal thymidine exposure.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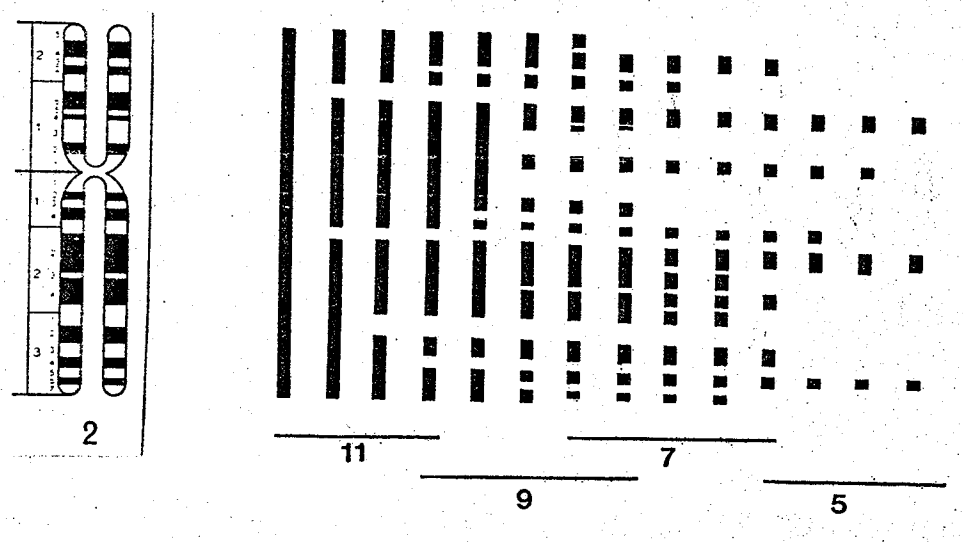
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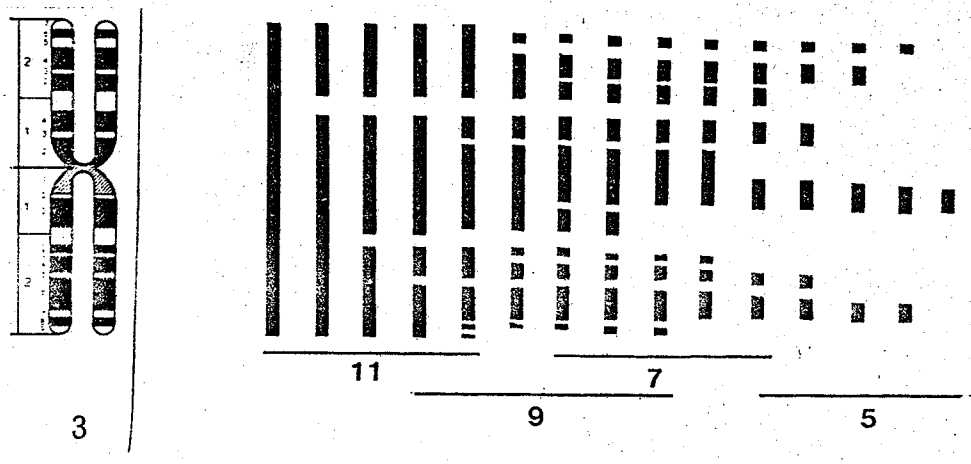
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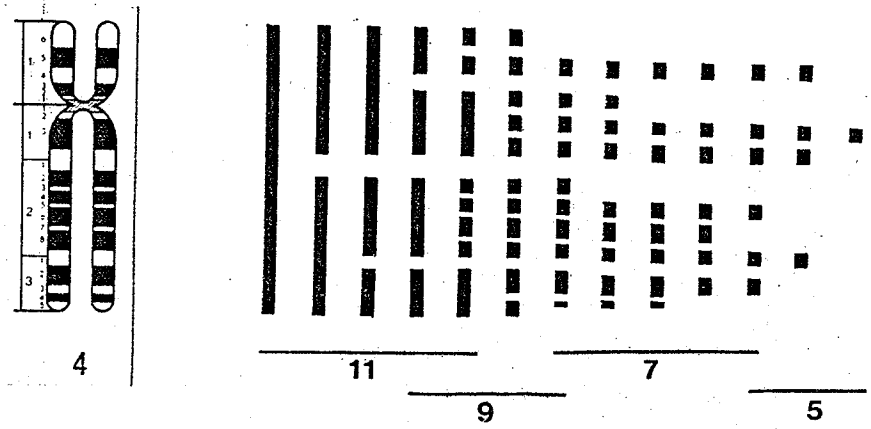
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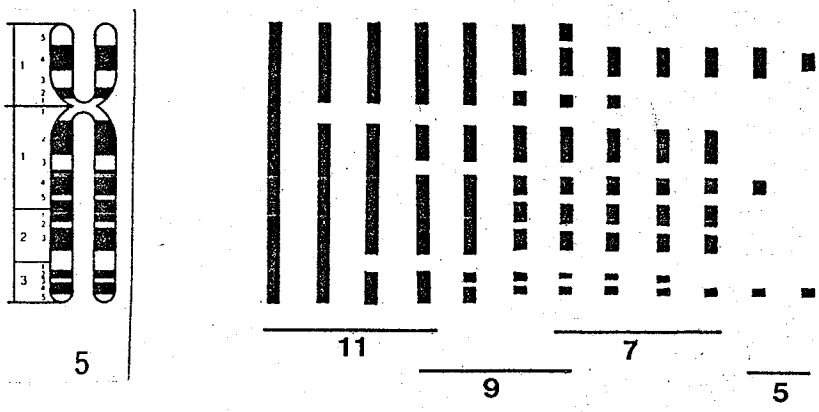
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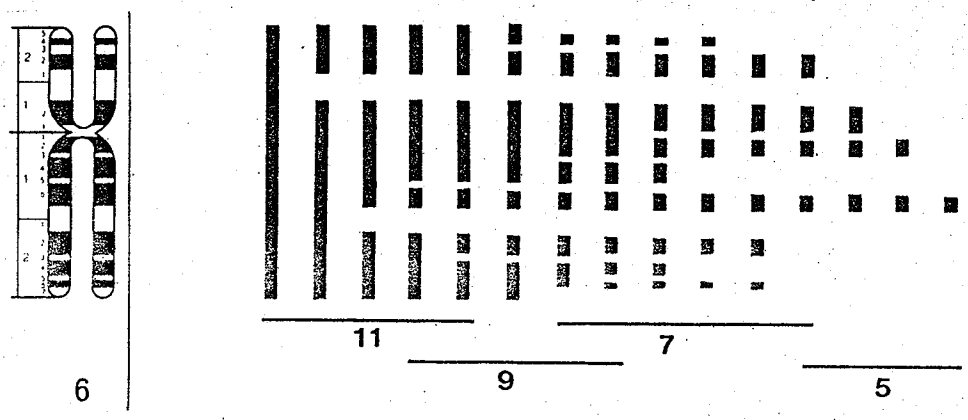
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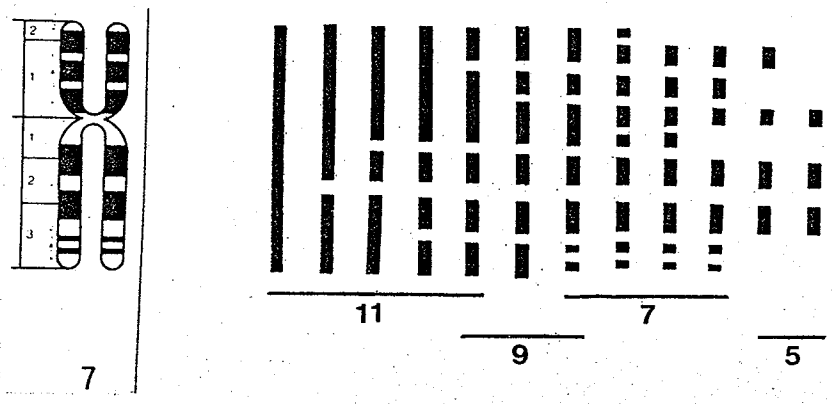

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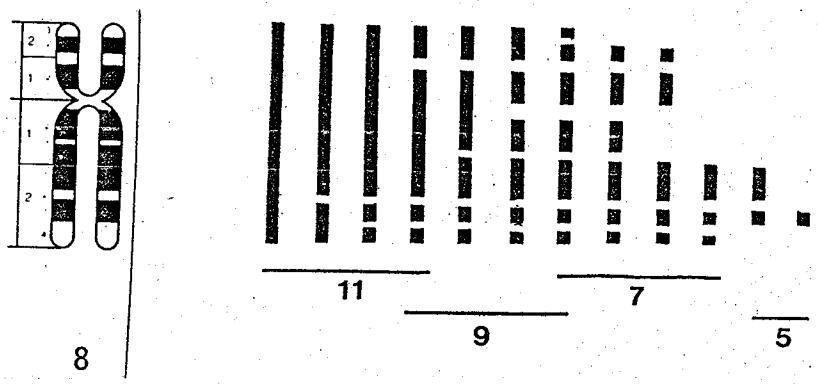

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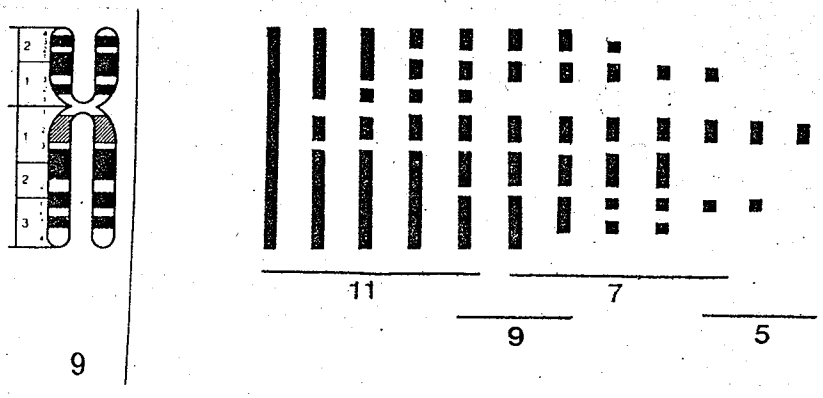

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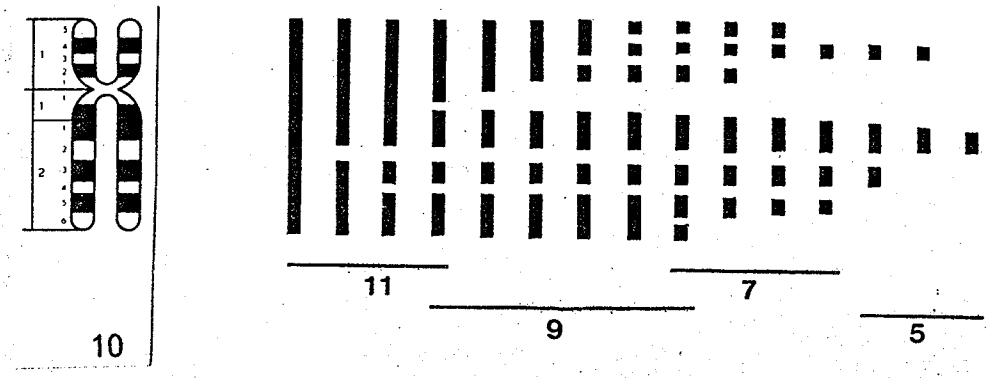
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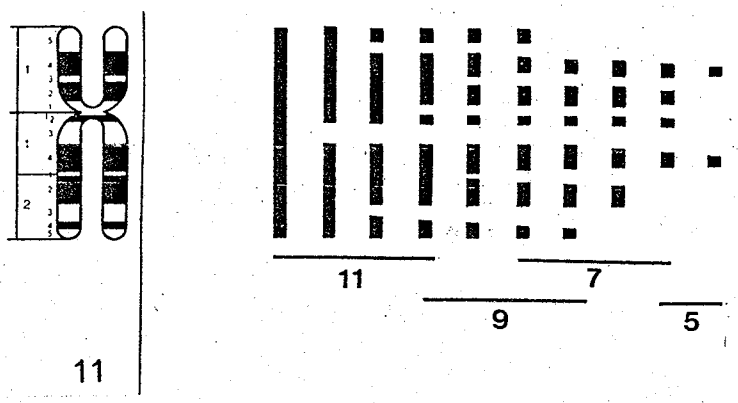
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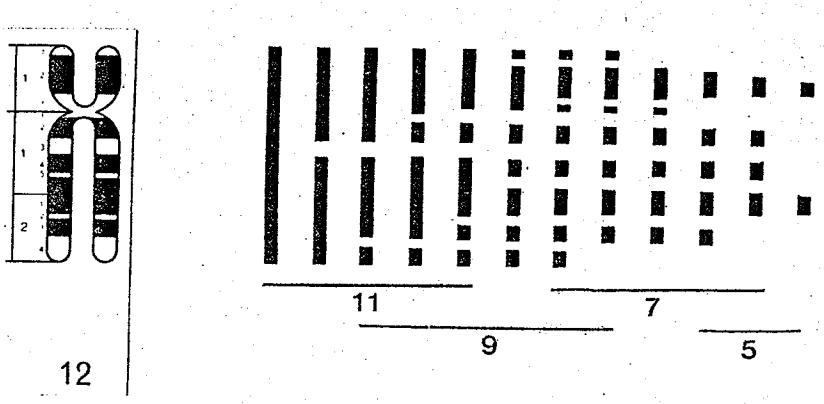
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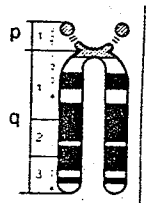

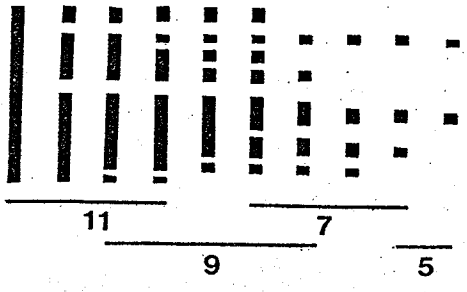

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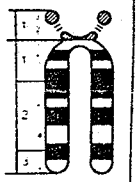

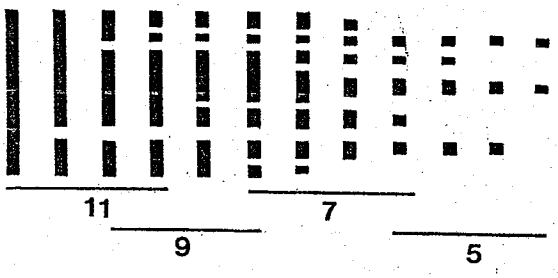

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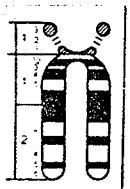

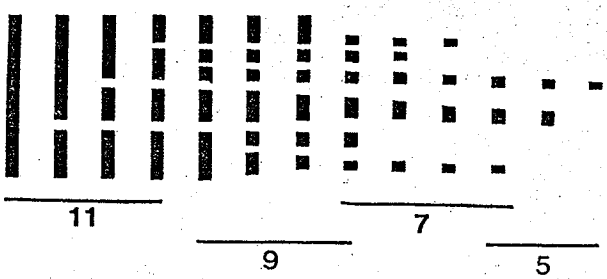

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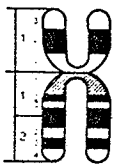

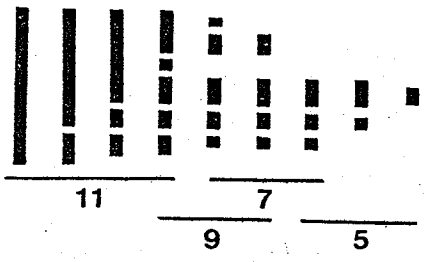

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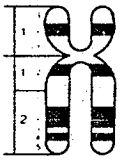

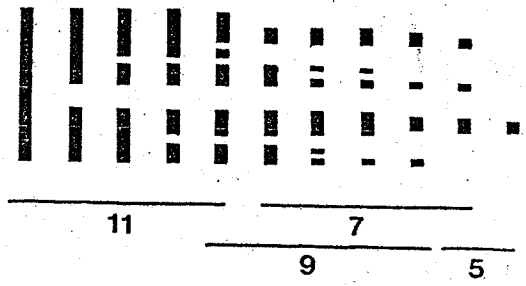

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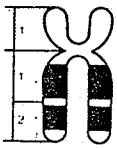

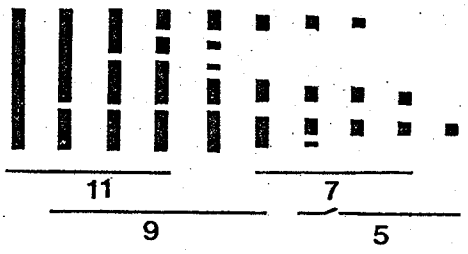

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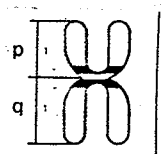

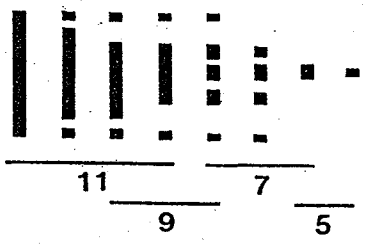

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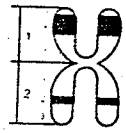

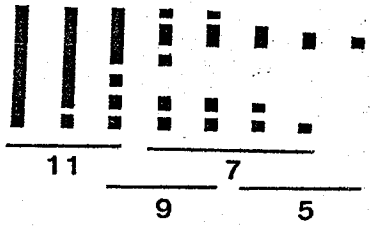

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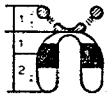

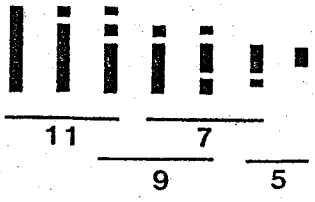

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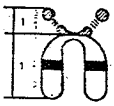

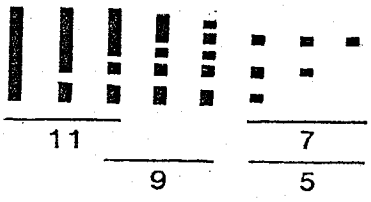

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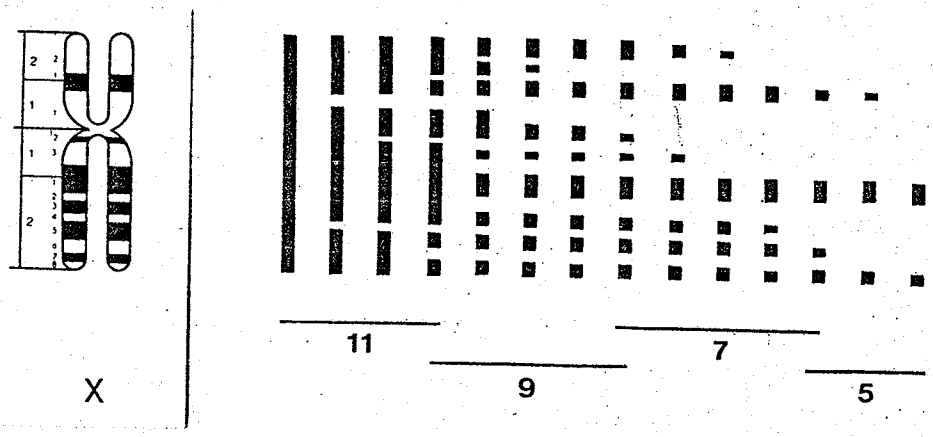

X
11
9
7
5

some was found to stain homogeneously dark when thymidine was incorporated for the entire S phase of that particular cell. As the duration of incubation in thymidine progressively decreased, the staining intensity of the chromosomes concurrently decreased and gaps in the staining began to appear. These regions represent the earliest areas to complete, and presumably initiate, DNA synthesis. As these gaps widen and increase in frequency, this results in first, the appearance of Q- or G-banding pattern and finally in a C-banding pattern.

It should be emphasized that although the banding patterns observed in each treatment group in some instances overlap, this is to be expected because of the unsynchronized cell population. Also, this demonstrates that DNA synthesis is a continuous process throughout the S phase and does not occur in discrete sharply defined stages.

As the terminal thymidine pulse was shortened, the percentage of cells in which the early and late replicating X chromosomes were differentiated steadily increased (Table 6). Only 17% of the homologous X chromosomes were distinguishable in the 11 hour group primarily by their differential overall staining intensity. In contrast, 71% of the X chromosome pairs in the 5 hour treatment group were distinguishable by their differential staining.

In the cases where the homologous X chromosomes were differentiated, the differential staining was dependent upon the length of exposure to thymidine (Table 7). The isocyclic X chromosome in the 9 hour group was consistently distinguished from the heterocyclic X primarily by two

Table 6. Percentage of differentially Giemsa replication stained homologous X chromosomes steadily increased with decreased terminal thymidine exposure.

TABLE 6

FREQUENCY OF DIFFERENTIATION

BETWEEN HOMOLOGOUS X CHROMOSOMES

DURATION TERMINAL THYMIDINE PULSE (HR)	CELL NUMBER	DIFFERENTIALLY STAINED
11	30	17%
9	30	24%
7	27	56%
5	30	71%

Table 7. In the 9 hour group the isocyclic X was differentiated from its homologue by decreased staining intensity of the short arm which in 34% of early replicating X chromosomes involved the complete absence of a band at pter. In the 7 hour group this decreased staining intensity of the isocyclic X was also observed at q23 and q27.

TABLE 7

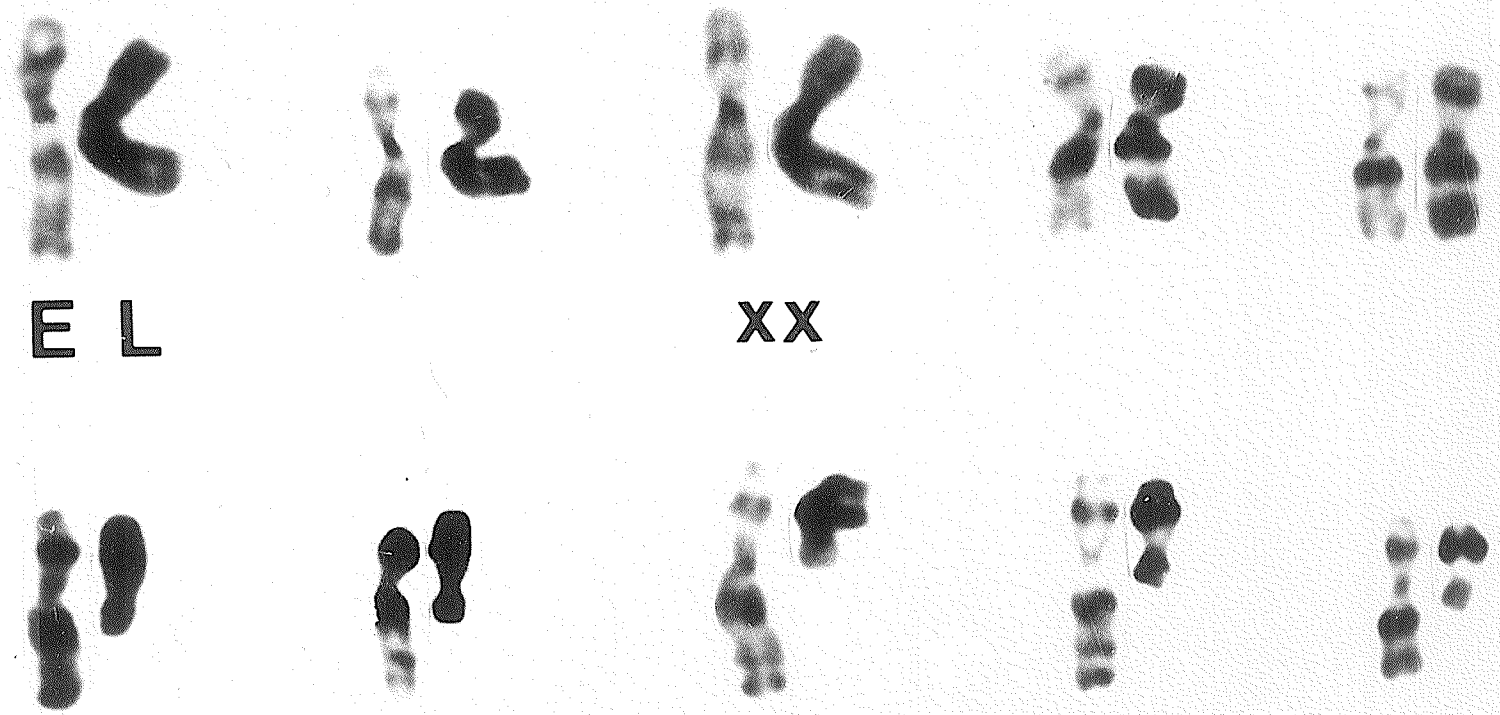
DIFFERENTIAL STAINING X CHROMOSOMES

THYMIDINE PULSE (HOURS)	PERCENTAGE OF ISOCYCLIC X WITH BANDS AT						HETEROCYCLIC X UNIFORMLY DARK (%)
	pter	p21	q21	q23	q25	q27	
9	66	100	100	100	100	100	100
7	33	100	100	33	100	66	90

characteristics. The early X stained less intensely overall than the late X and the short arm of the early X particularly near the telomeric region was stained noticeably lighter than the long arm. In fact, in 34% of such homologous pairs no band was observed at pter of the isocyclic X. However, in the 7 hour group not only was this decreased staining observed in the short arm in 67% of homologous pairs but also was visible at two regions of the long arm, q23 and q27.

In approximately 10% of the metaphases from the 7 hour terminal thymidine pulse, the heterocyclic X chromosome did not stain uniformly dark but had a banded appearance (Fig. 11). Conspicuous bands are present at p21, q21, q25, and q27. The late X of each homologous pair stains noticeably darker than its homologue in addition to having either more bands present or wider bands. Fig. 11 also demonstrates that the X chromosome with a deletion at q13 was consistently late replicating in all cells where both were identified. Although all of these homologous pairs were obtained from the 7 hour treatment group, they show a gradient of DNA replication. The pairs for both the normal 46,XX, and the abnormal 46,X,del(X)(q13) line on the left demonstrate earlier areas of DNA synthesis staining than the pairs on the right. The heterocyclic X chromosome from the normal diploid cell line on the left of figure has late DNA replicating bands present at pter and q23. These regions have completed DNA synthesis before the addition of thymidine in the heterocyclic X on the right of figure and therefore only the latest DNA replicating areas such as p21 remain banded. This same pattern of late DNA synthesis was visible on the partially deleted

Figure 11. Homologous X Chromosomes of Diploid and Xq- Cell Lines from the 7 hour terminal thymidine groups. Early replicating X is on the left of each pair while the late replicating X is on the right of each pair.



7 HOURS

heterocyclic X chromosome with p21 also remaining the latest DNA replicating region of the short arm.

In order to demonstrate the heteropycnosis of the heterocyclic X chromosome during the interphase, the frequency of sex chromatin was determined in both cell lines with Cresyl violet and Acridine orange (Table 8). A significantly larger percentage of the total nuclei, 60%, were sex chromatin positive in the normal cell strain after staining with Acridine orange as compared to 25% positive after staining with Cresyl violet. This improvement resulted from an increased resolution of cell nuclei afforded by Acridine orange (Figures 12, 13, 14, 15). The sex chromatin body appeared as a brightly fluorescent green mass primarily located at the periphery of the nucleus. The nucleoli stained bright red which precludes them from being mistakenly identified as sex chromatin bodies. The high degree of cytological differentiation observed made possible the identification of the sex chromatin even when located in the center of the nucleus.

When the size of the sex chromatin body is compared either with Cresyl violet staining (Figures 12, 13) or with Acridine orange (Figures 14, 15), the size of the sex chromatin body is significantly decreased in the fibroblasts with a deletion of the long arm of the X chromosome. This provides additional evidence for the genetic inactivity and hence late replication of the partially deleted X chromosome.

Table 8. In the 46,XX, fibroblast strain, Acridine orange increased the frequency of the sex chromatin identification due to the increased resolution of this technique. Similar explanation may indicate why fewer 46,XXq-, nuclei were sex chromatin positive after staining with Acridine orange.

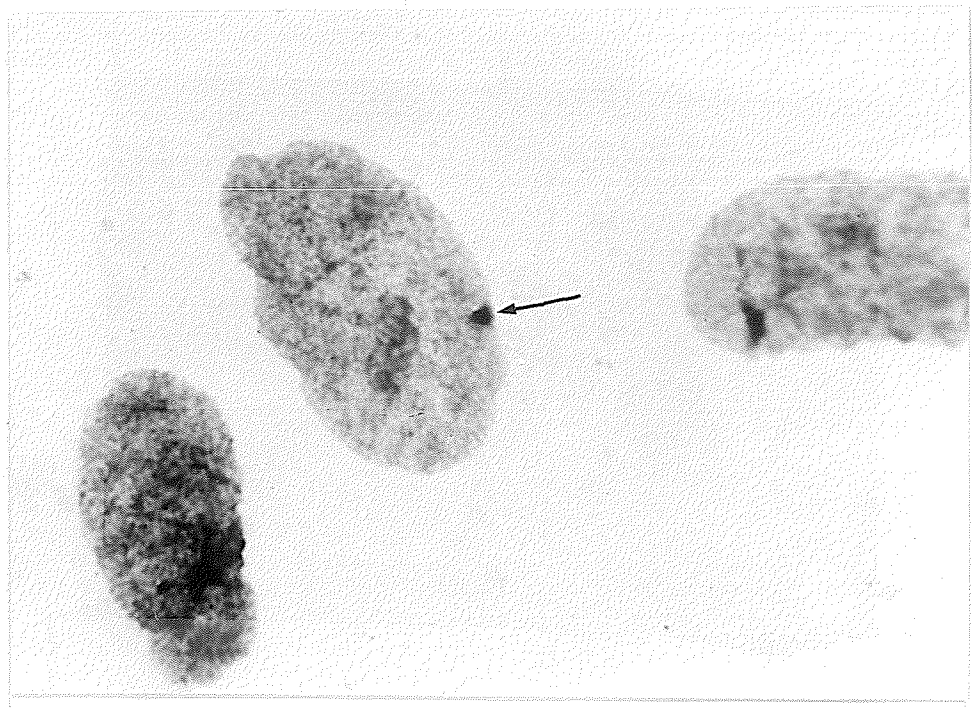
TABLE 8

IDENTIFICATION SEX CHROMATIN

STAIN	46 XX		46 XXq-	
	CELL NUMBER	PER CENT POSITIVE	CELL NUMBER	PER CENT POSITIVE
CRESYL VIOLET	631	25	613	5.2
ACRIDINE ORANGE	421	60	435	3.2

Figure 12. (upper) Deleted X Sex Chromatin Identified with Cresyl violet (indicated with arrow).

Figure 13. (lower) Normal X Sex Chromatin Identified with Cresyl violet (indicated with arrow)



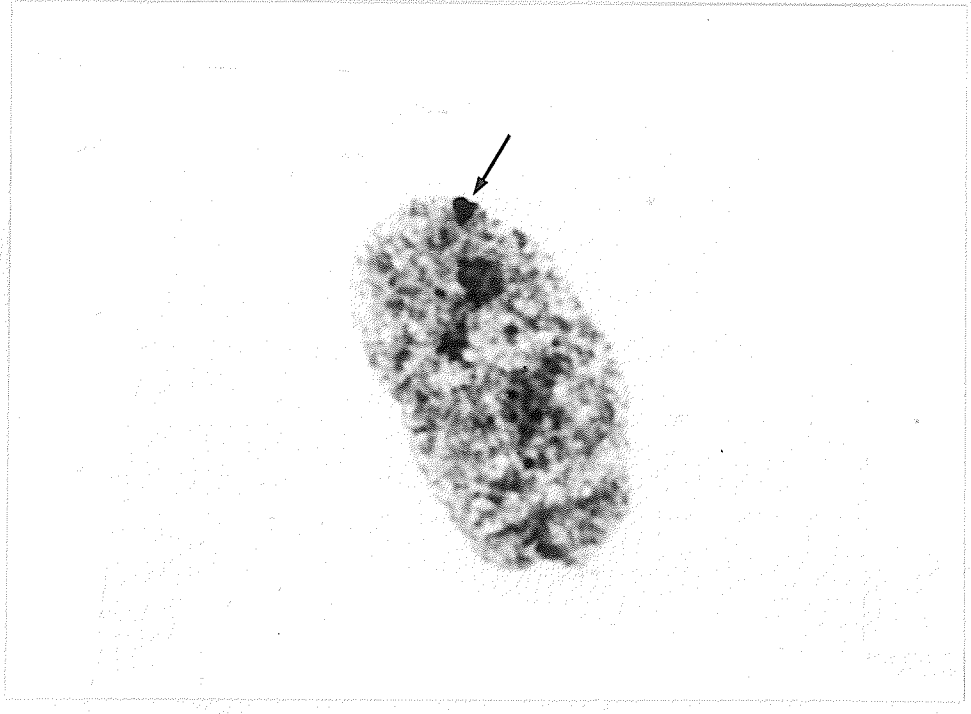
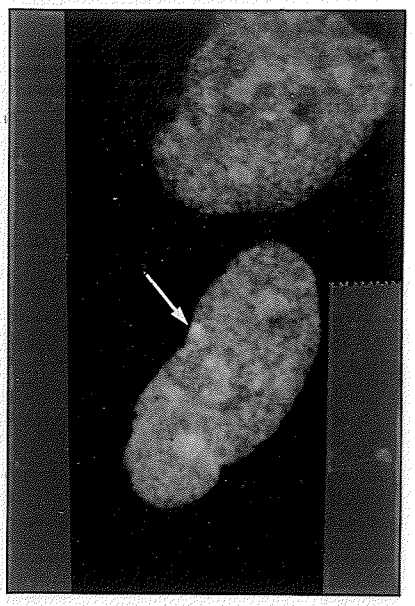
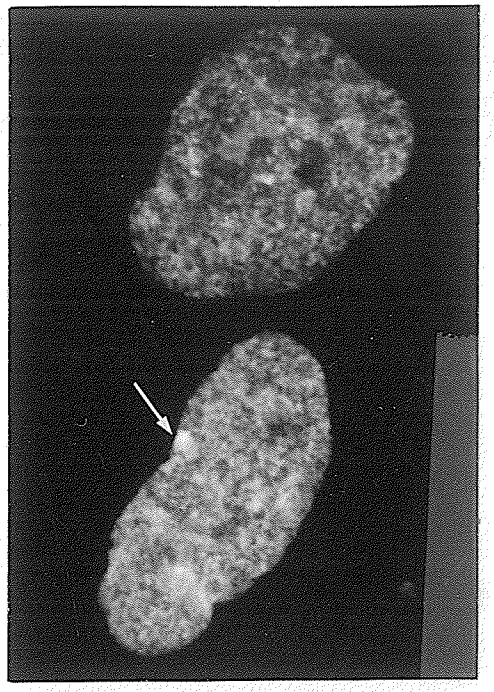
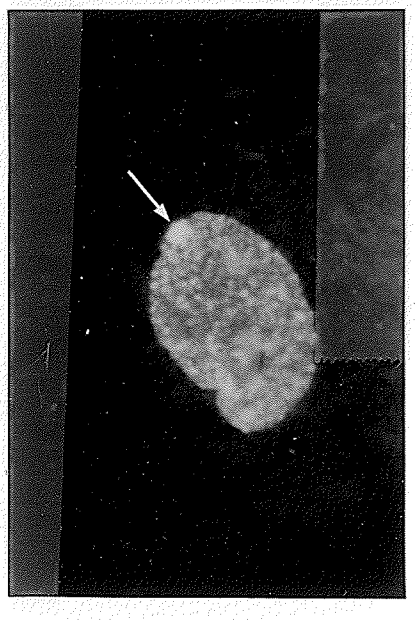


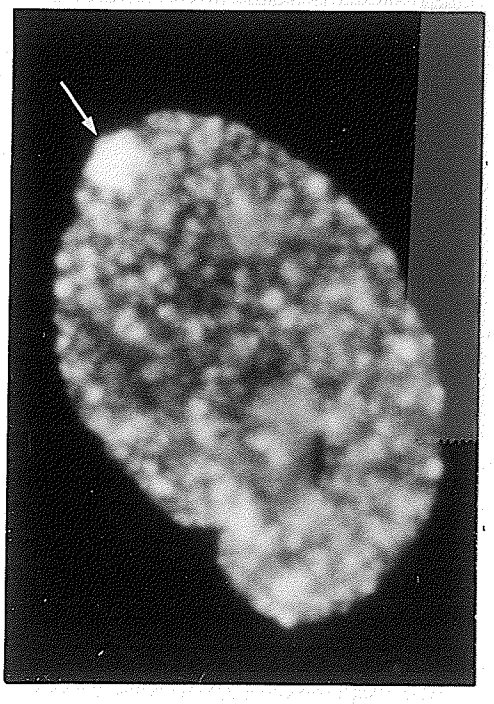
Figure 14. (upper) Deleted X Sex Chromatin Identified with Acridine orange (Indicated with arrow). Printed in both black and white and color.

Figure 15. (lower) Normal X Sex Chromatin Identified with Acridine orange (Indicated with arrow). Printed in both black and white and color.









DISCUSSION

From the initial experiments involving the continuous culturing of fibroblasts for 3 or more S phases (4 days or more) the sensitivity of cell strain SPF 46,XX to BrdU became apparent. BrdU reduced the mitotic index of these fibroblasts to a negligible level even at the concentration of 1.5 µg/ml (Table 4). However, the inhibitory effect of FrdU in addition to BrdU upon cell division was sufficient to completely prevent cell division (Table 4, Experiments 12-15). This was in spite of the addition of uridine to overcome the inhibition of nucleotide synthesis by FrdU.

In the metaphases obtained differentially stained sister chromatids (Fig. 2) were consistently observed after FPG staining whereas banded chromosomes were not present. When the molecular basis of the FPG differential staining was considered it became apparent that these differentially stained sister chromatids represented cells which had undergone only 2 S phases in BrdU instead of the expected 3 or more. Presumably this resulted from the cytotoxicity of BrdU because even if the incubation time was increased from 4 to 7 days (before terminal thymidine pulse), only differentially stained chromatids were observed. As a result of this high concentration of BrdU the cell cycle time was increased and these cells were unable to undergo any further growth and DNA synthesis.

After incubation of the fibroblasts for one S phase in BrdU the DNA at the subsequent metaphase was unifilarly substituted with BrdU (Fig. 16). When these cells were grown in BrdU enriched media during the second S phase, at the second metaphase one chromatid of each chromosome was unifilarly substituted (Fig. 17). However the remaining chromatid was

Figure 16. Effect of Bromodeoxyuridine Incorporation Upon Staining of Chromosomes (1 S Phase).

FIGURE 16

EFFECT OF BROMODEOXYURIDINE INCORPORATION

UPON STAINING OF CHROMOSOMES

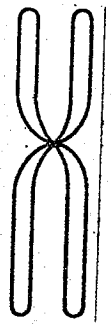
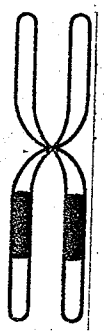
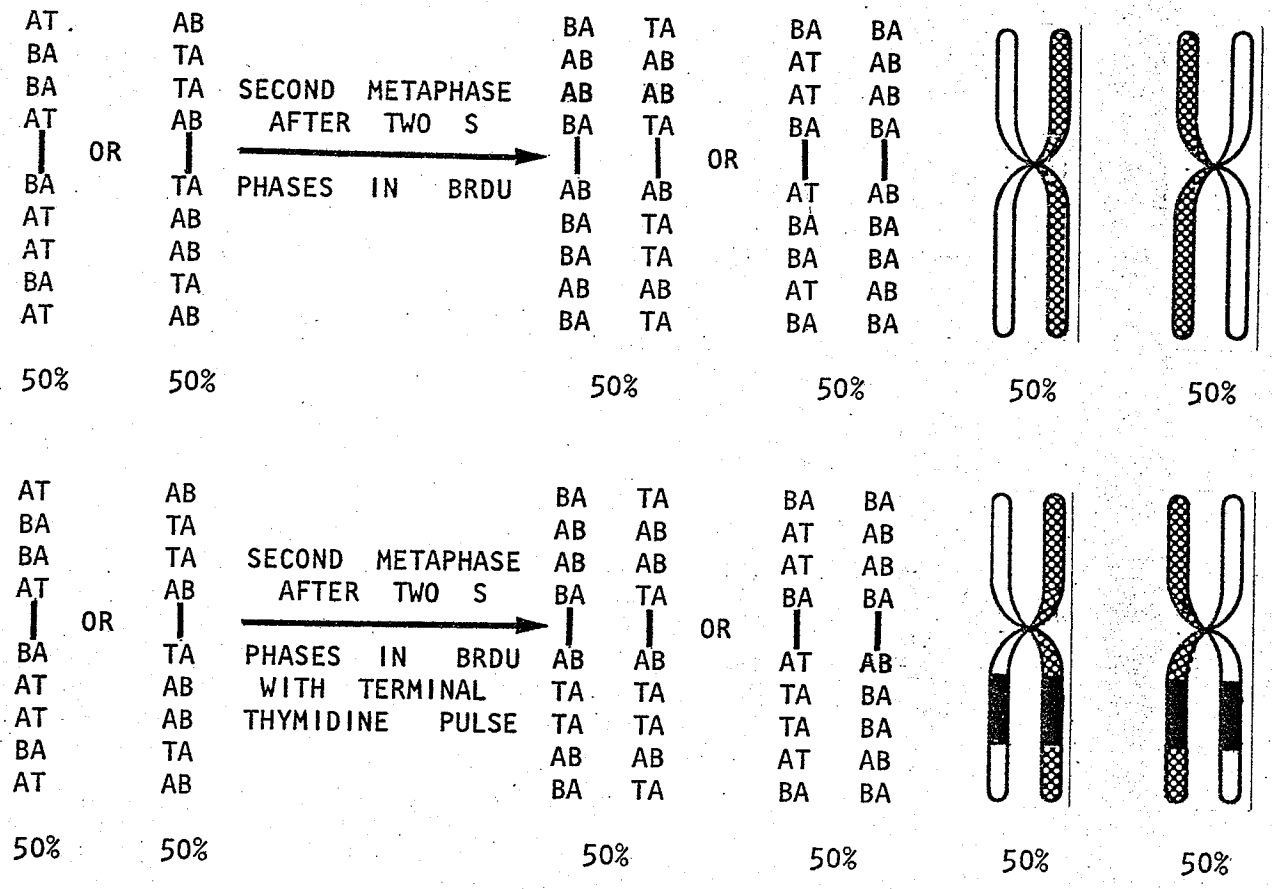
INTERPHASE		MOLECULAR LEVEL	CYTOLOGICAL LEVEL
TA AT AT TA | AT TA TA AT TA 100%	AT FIRST METAPHASE AFTER → ONE S PHASE IN BRDU	AT AB BA TA BA TA AT AB | BA TA AT AB AT AB BA TA AT AB 100 %	 100 %
TA AT AT TA | AT TA TA AT TA 100%	AT FIRST METAPHASE AFTER → ONE S PHASE IN BRDU WITH TERMINAL THYMIDINE PULSE	AT AB BA TA BA TA AT AB | BA TA AT AT AT AT BA TA AT AB 100 %	 100 %

Figure 17. Effect of Bromodeoxyuridine Incorporation Upon Staining of Chromosomes (2 S Phases).

FIGURE 17

EFFECT OF BROMODEOXYURIDINE INCORPORATION
UPON STAINING OF CHROMOSOMES



bifilarly substituted. After staining of these chromosomes with FPG technique this resulted in sister chromatid differentiation of all cells which had progressed through 2 S phases in BrdU.

Occasionally differentially stained and banded chromatids were observed (Fig. 3). This must have resulted from a terminal thymidine pulse during the second S phase in BrdU (Fig.17). The chromatid differentiation resulted from unifilarly and bifilarly substituted sister chromatids. However, the regions of chromosomes which had undergone DNA synthesis during the terminal thymidine pulse incorporated this base and therefore stained more intensely with the FPG technique. Consequently, this resulted in the cytological observation of bands superimposed upon that of chromatid differentiation. The staining produced by either Giemsa replication banding or differentiation of sister chromatids is quite similar and can be mistaken. Therefore such chromosomes were unsuitable for replication mapping purposes.

From these experiments it was concluded that the cytotoxicity of BrdU was sufficient to prevent the growth of the majority of these fibroblasts beyond 2 S phases. Subsequently, it was decided to decrease the exposure to BrdU to 1 S phase with a terminal thymidine pulse (Fig. 16). These chromosomes were unifilarly substituted with BrdU as opposed to complete bifilar substitution after 3 or more S phases of incorporation of BrdU. When a terminal thymidine pulse was administered after 1 S period in BrdU, any DNA synthesizing chromosomal regions would not be substituted with bromodeoxyuridine (Figure 16). This

difference in level of BrdU incorporation resulted in differential staining and hence the production of bands. These regions have an increased staining intensity with the FPG technique when compared to unifilarly substituted regions. Therefore, banding resulted from the different staining characteristics of unsubstituted as compared to unifilarly BrdU substituted chromosomal regions. Korenberg and Freedlender (1974) reported that the cytological differentiation obtained with this differential in BrdU substitution was less than that from bifilarly and unifilarly BrdU substituted chromosomes. However, when their FPG technique was applied to these chromosomes obtained after 1 S phase in BrdU and thymidine, sufficient differentiation resulted which permitted replication mapping. Equally importantly, a high mitotic index was obtained with this modified technique which made replication mapping feasible.

When these chromosomes were stained with the SSC-FPG procedure of Perry and Wolff (1974) and Wolff and Perry (1974) the resulting cytological differentiation was not comparable to that obtained after staining with Korenberg and Freedlender's (1974) FPG technique. In addition, the SSC protocol has been reported to produce a background staining of G-bands (Grzeschik et al., 1975). This may be critical in the determination of an accurate replication map. Also, the SSC-FPG technique was not as reliable as the NaH_2PO_4-FPG procedure which consistently produced reliable banding.

The quality of Giemsa replication bands obtained resulting from either Quinacrine or Hoechst prestaining were compared. Quinacrine

was essential for the identification of chromosomes. No difference in Giemsa replication (GR) banding was observed when either Quinacrine or Hoechst fluorescent prestaining was performed. However, when these Q-banded chromosomes were photographed they either failed to stain with Giemsa while other metaphases on the same slide had highly differentiated GR-bands or were removed from the slides as a result of incubation with 1 M NaH_2PO_4 (pH 8.0) at 84-85°C for 10 minutes. When the exposure to fluorescent light was decreased by shortening the duration of photographic exposure faint GR-bands appeared. Similarly, when the light source was changed to a 15 watt U.V. light instead of the Osram HBO 200 watt/4 Super Pressure mercury light on the fluorescent microscope, sharp GR-bands resulted. Therefore to achieve optimal GR-banded chromosomes overnight exposure to 15 watt U.V. light source at a distance of 24" was chosen. Because sequentially Q and GR stained chromosomes were essential for conclusive chromosome identification, especially of the C group, destaining of the GR-banded chromosomes and restaining with Quinacrine was attempted. This yielded banded chromosomes with sharply defined standard Q-bands. This reverse technique offered several advantages. Metaphases were selected on the basis of the quality of their GR-bands as opposed to Q-bands. Often sister chromatid separation was masked by flare from the fluorescent stained chromosomes which on subsequent banding had unsuitable GR-bands. Scanning of slides was simpler and faster using light microscopy. Finally, even weakly fluorescing metaphases were photographed with long exposure time without regard to the quality of GR-bands which had been photographed previously. This

procedure proved to be the most efficient for the sequential staining of Q- and GR-banded chromosomes.

These various staining procedures generated information regarding the mechanism of Giemsa replication staining. Several models have been postulated to explain the effects of BrdU incorporation upon subsequent Giemsa staining and Hoechst fluorescence. Zakharov and Egolina (1972) theorized that BrdU caused an alteration of the DNA molecule thereby impairing the synthesis of chromosomal condensing proteins and causing differential spiralization and Giemsa staining of BrdU substituted segments. Ikusima and Wolff (1974) postulated that the difference in binding of proteins to BrdU containing chromatids was the source of structural differentiation and therefore, the cause of differential staining with FPG procedure. Both Zakharov and Egolina (1972) and Ikusima and Wolff (1974) viewed with great importance the effect of BrdU upon chromosomal proteins. Because both urea and trypsin, which markedly alter the binding of chromosomal proteins, failed to reverse this differential Giemsa staining, proteins are not believed to be significant in producing this differentiation (Goto et al., 1975; Shiraishi and Yosida, 1972; Sugiyama, 1968, Sugiyama et al., 1976). The mechanism proposed by Comings (1975) supported this concept. Hoechst is believed to bind directly to the outside of the DNA helix by interacting with the base pairs. This type of binding results in greater sensitivity to the base composition than occurs with intercalating agents. Therefore, any base substitution would directly effect the binding, and hence the fluorescence, of Hoechst producing

bands (Comings, 1975).

The results of Goto et al. (1975) and Sugiyama et al. (1976) indicated that the photolysis of BrdU substituted DNA was the predominant cause of differential staining. This mechanism explains the observation of Perry and Wolff (1974) which was confirmed by the present investigations. When metaphase chromosomes were exposed to an intense level of exciting light during the process of photographing Q-banded chromosomes, subsequent Giemsa differentiation of chromatids was very weak and indeed, was not observed in many instances. The final staining protocol, as outlined in the Materials and Methods Section, proved to be reliable and enabled the photographing of sequentially Q- and GR-banded chromosomes.

Kim et al. (1975) reported that the early replicating regions coincide with bands produced by the R-banding technique (Dutrillaux and Lejeune, 1971). The results presented (Table 5), in general, confirm this. The reason that Kim et al. (1975) reported more early replicating bands than those presented in Table 5 is primarily due to the different experimental protocols employed. The early replicating segments of Table 5 were identified as gaps in the banding of chromosomes from the 11 hour treatment group while Kim et al. (1975) visualized early replicating DNA by the reverse experimental protocol which yielded bands. This could be expected to yield a greater number of early DNA synthesizing regions due to the increased sensitivity of this reverse experimental protocol.

The bands obtained following a 9 hour thymidine pulse were

similar to those observed after standard Quinacrine or Trypsin Giemsa staining. However, several additional replicating bands were stained. In addition to those bands indicated in Table 9 for the 7 hour treatment group, the telomeric regions of chromosomes 16p and 19q are late replicating although G- and Q- negative.

Following a 7 hour thymidine pulse the bands observed closely approximate Q- and G- bands. The additional late replicating bands which are G- and Q- negative are indicated in Table 9. Several Q- and G- bands replicate earlier in the S phase than the majority of Q- and G- bands such as a portion of bands 3p14 and 3q13, the centromeric heterochromatin of chromosome 8, and 9p12. A comparison of late replicating bands between Epplen et al. (1975), Epplen and Vogel (1975), Grzeschik et al. (1975) and these results revealed several contradictory bands (Table 10). The late replicating bands of chromosomes 2, 7, 12, 13 and 16 are identical in the case of Grzeschik et al. (1975) and these results, while the late DNA synthesizing segments of chromosome 6 are more similar in these results and those of Epplen and Vogel (1975).

When the terminal thymidine pulse is decreased to 5 hours, one of the most frequently observed banding patterns is the characteristic C-bands. These heterochromatic regions have been shown by autoradiographic techniques (Lima de Faria, 1969) and by Giemsa replication procedures (Grzeschik et al., 1975; Epplen et al., 1975; Epplen and Vogel, 1975) to replicate very late in the S phase of the cell cycle. These results confirm this observation with the heterochromatic

Table 9. Bands listed remain unstained after G- or Q- banding procedures but were determined to be late replicating from the 7 hour terminal thymidine group.

TABLE 9

G- AND Q- NEGATIVE LATE REPLICATING BANDS*

CHROMOSOME	BAND
1	pter
2	p25, p15
3	p23, q12
5	p15
6	q25
7	p22, p11, q11
8	p23, q24
9	p24
10	p15, q26
11	pter
12	qter
13	q12
14	q32
17	q11, q25
18	pter
20	q12, qter
21	qter
X	pter

* Taken from 7 hour terminal thymidine group

Table 10. Bands listed for any author which were conflicting with the late replicating bands of any other are indicated. Unlisted chromosomes had no significantly different pattern of replication.

TABLE 10

DISCREPANCIES OF LATE REPLICATING BANDS OF HUMAN CHROMOSOMES

CHROMOSOME	EPPLEN ET AL., 1975* EPPLEN & VOGEL, 1975*	GRZESCHIK ET AL., 1975**	PRESENT*** RESULTS
1	p36, p33, q22, q32	q31, q41	p33, q31, q41
2	q31	q24, q32, q36	q24, q32, q36
3	q28	p14, q13	p14
6	p24, q14	--	p24, q14
7	--	q11	q11
8	p21, qter	qter	--
12	q24	--	--
13	--	q33	q33
14	--	q23	q23
16	p13	p12	p12
17	--	p12, q12, q24	p12, q12, q25
18	--	--	pter
19	q12, q13	--	q12
20	q11, q13	p12	p12, q12, q13

* Amniotic Fluid Cells ** Lymphocytes *** Fibroblasts

regions on chromosomes 1, 3, 4, 9, 13, 14, 15, 16, 21, and 22 replicating consistently very late as evidenced by the 5 hour treatment group. The late replication map of the 46,X,del(X)(q13) fibroblast strain was determined not to significantly differ from the normal 46, XX cell strain. This is consistent with the concept that the replication of each chromosome is independently regulated.

The terminal DNA replication kinetics of human chromosomes in human lymphocyte/mouse fibroblast hybrids were studied (Lin and Davidson, 1975). The sequence of late DNA replication was determined to be the same in hybrids as in the corresponding chromosomes in human lymphocytes by autoradiographic techniques. Also, the maintenance of normal terminal replication sequences of human chromosomes in these hybrids was not dependent upon the presence of any specific human chromosome. The present results fully support this conclusion.

The differential staining of the homologous X chromosome is primarily determined by the timing of the addition of thymidine to the cell culture (Table 6). In the 11 hour treatment group the majority of homologous X chromosomes, 83% of the cells where both were identifiable, had not initiated DNA synthesis prior to the addition of thymidine. Consequently both homologues incorporated thymidine along their entire length and stain uniformly dark with the FPG procedure. In the 17% of the cells where the isocyclic X and heterocyclic X were distinguishable, this differentiation resulted from the addition of thymidine when the isocyclic X had completed the replication of a portion of its DNA while the heterocyclic X had not initiated DNA synthesis. Consequently,

this results in differential BrdU/thymidine substitution with the late X incorporating thymidine and therefore staining darker with the FPG technique (Fig. 11).

The intrachromosomal replication pattern of both X chromosomes presented in Fig. 11 was found to be very similar. The variously banded isocyclic and heterocyclic X chromosomes were readily ordered to derive their replication map. However, the initiation and completion of DNA synthesis differed with the inactive heteropycnotic X replicating later. This is in agreement with autoradiographic analyses which have established that the inactive X can be distinguished from its active homologue by a delay both in the initiation and termination of DNA synthesis (Priest et al., 1967 b; German, 1964 a). Willard and Latt (1976) also reported that the internal replication kinetics of homologous X chromosomes are similar with the exception that DNA synthesis of band q23 is delayed in the late X while DNA synthesis of band q27 is delayed in the early X. Grzeschik et al. (1975) found no consistent pattern of delayed replication of bands q23 or q27 but reported considerable fluctuation in the timing of DNA synthesis of these bands and the replication kinetics of the X chromosomes in general.

Additional evidence for the late replication of the heteropycnotic inactive X chromosome was derived from the fibroblast strain SK5239. The structurally abnormal X chromosome with a deletion at q13 was consistently late replicating (Fig. 11) and the nuclei of this fibroblast strain showed consistently smaller sex chromatin mass than

the normal diploid fibroblast strain (Fig. 12, 14). This is in agreement with autoradiographic results which indicated that the structurally abnormal X chromosome was invariably late replicating (Gianelli, 1970; Fraccaro and Lindsten, 1964; Atkins et al., 1965; Steinberger et al., 1966). The constancy of the inactivation of the structurally abnormal X chromosome may be a result of cell selection. Cells with an active deleted X may not have either the reproductive potential nor the life span and may not proliferate as readily as the converse case.

The consistent genetic inactivation of the deleted X chromosome provides further evidence regarding the models of X chromosome inactivation in female eutherian mammals. Grumbach (1964), Lyon (1964), Russell (1964), and Therman et al., (1974) proposed that X chromosome differentiation in mammals is under the control of a single site or locus within the X. The concept has developed that an inactivation center within the X exists from which some substance spreads to inactivate the entire X chromosome. These results indicate that there cannot be one inactivation center on the long arm of the X chromosome as proposed by Therman et al.(1974). The consistent inactivation of the deleted X chromosomes strongly suggests that if this model is correct, several inactivation centers must be present. Cattanach (1975) reviewed the results of several investigations which seriously question this hypothesis of X chromosome inactivation.

Although the previously mentioned hypothesis is based on the premise of inactivation rather than activation, several models propose

the converse mechanism. Comings (1968) provided evidence that chromosomes are attached to the nuclear membrane with the attachment sites possibly corresponding to points of initiation of DNA synthesis. He proposed that only one such site may exist for the X chromosome and additional X chromosomes not attached to these sites might contract and attach randomly to the nuclear membrane where they might replicate later than the rest of the chromatin in the cell. This concept is compatible with the results obtained from this study.

The hypothesis of Brown and Chandra (1973) and Chandra and Brown (1975) is also based upon the premise of activation of the X chromosome rather than inactivation. Basically, their model requires that a receptor site on the X chromosome be activated by a single informational entity which results in the activation of the chromosome. If the receptor site necessary for the X chromosome activation is absent in the deleted X chromosomes, this would result in the inactivation of these structurally abnormal X's. The model of Brown and Chandra (1973) is consistent with these findings and other autoradiographic findings (Gianelli, 1970; Fraccaro and Lindsten, 1974; Atkins et al., 1965; Steinberger et al., 1966) which indicate that the structurally abnormal X chromosome was invariably late replicating.

These results could not conclusively resolve the contradictory replication kinetics between lymphocytes, amniotic fluid cells, and fibroblasts solely on the basis of a tissue specific replication map. These results from fibroblasts were found to be in agreement with either the lymphocyte or amniotic fluid replication map depending upon the

specific chromosome involved (Table 10). It is probable that several contradictory replication bands may be resolved on the basis of a tissue specific replication map. However, the differences observed may also be derived from the different experimental procedures employed and the fact that each study was performed on tissue from a different individual. When the tissues are derived from the same individual and similar experimental protocols are employed, valid results may be obtained which may demonstrate the existence of tissue specific replication maps.

The specificity and resolution of this technique indicates that several areas be investigated. In a synchronized cell population the accurate delineation of the duration of the S and G_2 phases of the cell cycle in a wide variety of cell lines with differing chromosome complements may be determined. As mentioned previously, this modified technique will permit the comparison of the replication kinetics of several tissues. In addition, similar comparisons could be made in cell hybrids with varying human chromosome complements which may provide further information regarding the independent control of chromosomal DNA synthesis. Transformed cell lines may differ in their replication pattern either from normal cells of the same tissue or from the same pre-transformed cell line. Investigations of lines with various deletions and translocations involving the X chromosomes will certainly generate new information regarding the replication patterns of the X chromosomes.

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