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

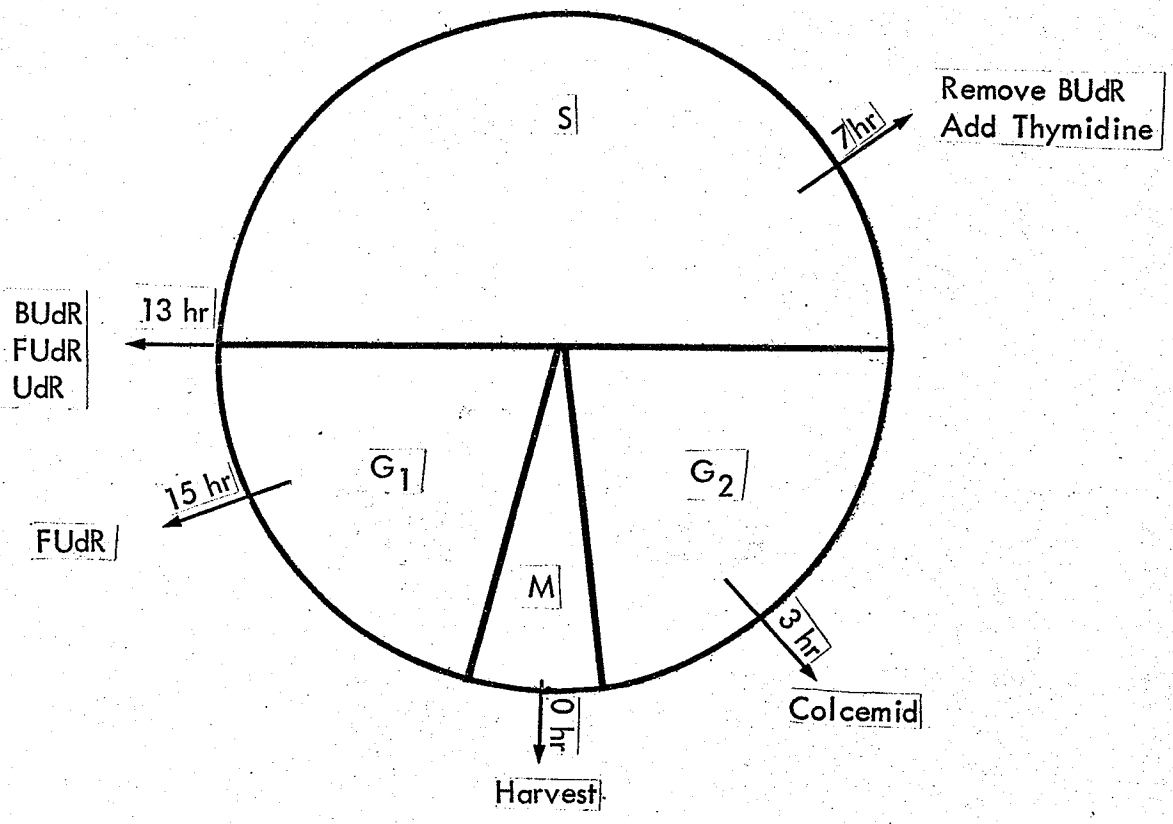

Table 1, (Defendi and Manson, 1963; Kukharenko et al., 1974; Moorhead and Defendi, 1963; Porter and Paul, 1974). Both the S and G_2 periods are less variable in duration than the G_1 in many different mammalian tissues and species (Aoki and Moore, 1970; Cleaver, 1967; Defendi and Manson, 1963; Gianelli, 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